University of Alberta

Immunotherapeutic Approaches for Prostate Cancer: Optimization of Antigens Targeting Dendritic Cells

By

Chun Piao ©

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Dedicated to

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Abstract

The aim of the work described is to develop a specific immunotherapeutic agent that can induce an effective immune response against prostate cancer. Optimization of modified proteins targeting to dendritic cells (DC) was studied, and the induction of the immune response by some of these modified proteins was investigated.

Prostate specific antigen (PSA), anti-PSA monoclonal antibody (MAb) and anti-DC MAbs were subjects of this study. Modifications of these proteins were conducted by mannosylation and mannan conjugation on PSA and anti-PSA MAb, and construction of bispecific agents with specific binding activities on PSA and DC by conjugation of anti-PSA MAb and anti-DC MAbs. Modified proteins were characterized by SDS-PAGE, silver staining, fluorescence microscopy, ELISAs, and FACS analysis. Induction of the anti-PSA immune response by bispecific agents was tested *in vivo*. Murine DC uptake capacities of ^{125}I labeled mannosylated and mannan conjugated anti-PSA MAb and PSA were tested and compared to nonmodified anti-PSA MAb and PSA *in vitro*.

Mannan conjugated anti-PSA MAb retained considerable PSA binding activity. Further evaluation on DC targeting and anti-PSA immune response induction need to be investigated to confirm its potential to reach the ultimate goal of this project.

Compared to nonmannosylated PSA, mannosylated PSA showed much stronger binding on the surface of DC in a fluorescence microscopy study. Similar in the DC uptake assay, an increased amount of mannosylated PSA was captured by dendritic cells. Mannosylated PSA could induce a stronger humoral anti-PSA immune response. Mannosylated or mannan conjugated PSA has a potential to reach the ultimate goal of this research.

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List of Abbreviations

Ab	Antibody
ABTS	2,2'-Azido-di [3-ethyl-benzthiazoline sulfonate]
APL	Altered peptide ligand
BPH	Benign prostate hyperplasia
BSA	Bovine serum albumin
Con A	Concanavalin A
CTL	Cytotoxic T lymphocyte
Da	Dalton
DC	Dendritic cells
DD	Double distilled
α-D-M	α-D-mannopyranosylphenylisothiocyanate
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal bovine serum
FDA	Food and Drug Administration
FITC	Fluorescein isothiocyanate
GM-CSF	Granulocyte/macrophage colony stimulating factor
HRP	Horse radish peroxidase
HBSS	Hank's balanced salt solution
HLA	Human leukocyte antigen
HPBMC	Human peripheral blood mononuclear cell
IFN γ	Interferon γ
Ig	Immunoglobulin
IgG	Immunoglobulin G
IL	Interleukin
IP	Intraperitoneal
KD	Kilodalton
MAb	Monoclonal antibody
MBP	Mannan binding protein

α-MEM	α-Minimum enssential medium
MHC	Major histocompatibility complex
MW	Molecular weight
NMM	N-methylmorpholine
PAP	Prostatic acid phosphate
PBMC	Peripheral blood mononuclear cell
PBS	Phosphate-buffered saline
PBST	PBS containing 0.01% tween 20
PSA	Prostate specific antigen
SCID	Severe combined immunodeficiency disease
SDS-PAGE	Sodium dodecylsulphate polyacrylamide gel electrophoresis
SFM	Serum free medium
TAP	Transporters associated with antigen processing
TNF	Tumor necrosis factor
UD	Ultradoma medium
UNLB	Unlabelled
UV	Ultraviolet

CHAPTER 1

Introduction

The incidence of prostate cancer appears to be declining, but it remains the most common malignancy in American men [Harris DT, 1999]. In the United States, prostate cancer is now the most commonly diagnosed cancer and the second most common cause of death from malignant disease in men, after lung cancer. In the year 2002, over 191,000 new cases of prostate cancer will be diagnosed and this cancer will cause over 31,000 deaths. It is estimated that one in every six American males will be diagnosed with prostate cancer sometime in their lifetime [Hillman GG, 1999]. The cost of prostate cancer treatment in the USA is also striking, in the annual range of $1 billion, with 250,000 hospitalizations [Hillman GG, 1999]. In Canada, it is estimated that about 18,200 new cases of prostate cancer will be diagnosed in the year 2002, ranking as the highest incidence among cancer cases. This will result in over 4,200 deaths, the second highest cause of cancer deaths [Information from *Canadian Cancer Statistics*, 2002].

The prostate is a sex gland found only in men. One of the functions of the prostate gland is to secrete prostatic fluid, which forms part of semen. The walnut sized prostate gland is composed of three distinct zones: the peripheral, central, and transition zones. The peripheral zone contains about 70% of the volume of the normal prostate gland, and is the most common site of prostatic intraepithelial neoplasia (PIN) and carcinoma [D'Amic AV, 1996].

Several grading systems have been developed for prostatic carcinoma, and many have shown a correlation with patient survival. "Gleason grading" is the most used by physicians and is recommended as standard [D'Amic AV, 1996]. This system is based entirely on the architectural features of the tumor and ignores cytologic features. Architectural patterns are grouped into five grades, numbered 1 to 5, with grade 1 having the best prognosis and grade 5, the worst [D'Amic AV, 1996; Bostwick DG, 1999]. Since the majority of tumors have more than one architectural pattern present, the grade of both the predominant architectural pattern as well as the second most common pattern are recorded. A Gleason sum is the sum of primary and secondary grades to create a Gleason score of 5 to 10. The presence of a primary Gleason pattern 4 or a Gleason sum of 7 is considered indicative of a poor prognosis [Patel RI, 2000].

The stage of a prostate cancer reflects the extent of the cancer. The commonly used staging systems are the ABCD staging system [Jewett HJ, 1975] and the TNM system [Aronson N, 1999]. TNM is becoming the preferred international staging system for prostate cancer [American Joint Committee on Cancer, 1975; Bostwick DG, 1999]. In 1992, the International Union against Cancer (IUCC)/American Joint Committee on Cancer (AJCC) proposed unifying revision of the TNM system for staging prostate cancer [Patel RI, 2000]. Clinical stage refers to an assessment of the tumor by digital rectal examination (DRE), serum prostate specific antigen (PSA), and radiologic imaging [Issa MM, 2000]. Most staging numbers reflect clinical staging other than pathologic staging, which is only possible after radical prostatectomy, the surgical removal of the prostate. The clinical staging system for prostate cancer is more complicated than the

grading system. It combines several parameters: PSA level, the clinical findings of a DRE, tumor volume, Gleason grade, transrectal ultrasound (TRUS), or the pathologic findings at transurethral resection of the prostate (TURP). Prostate cancer treatment is mandated by disease stage. Biologically, prostate cancer represents a heterogeneous disease entity with varying degrees of behaviour, aggressiveness, patterns of metastasis, and response to therapy [D'Amic AV, 1996]. Staging information and doctor's advice can guide and inform prostate cancer patients to make appropriate treatment decisions.

1.1. Conventional treatments for prostate cancer

Radical Prostatectomy

Radical prostatectomy is considered a very effective treatment for patients with localized prostate cancer and a life expectancy of 10 years or more [Shalev M, 2000]. It is a procedure to remove the entire prostate gland. Theoretically, if prostate cancer is localized to the prostate gland and not spread to the distant tissues, it can be cured by radical prostatectomy. It is important to point out that radical prostatectomy may cause a high incidence of serious side effects, such as impotence and urinary incontinence [Myers RP, 2000]. Treatment of localized prostate cancer by radical prostatectomy with PSA follow up testing indicates 70% to 80% freedom from PSA progression 10 years after surgery [Andriole GL, 1997].

Radiation therapy

Like surgery, radiation therapy works best when the cancer is caught at an early stage and is still confined to the prostate gland. External beam radiation therapy was first

introduced in 1956. This approach uses a beam of radiation that originates from outside the body [Bostwick DG, 1999]. Radiation acts at the cellular level to cause DNA damage within cells, leading to a loss of reproductive integrity and cell death. The goal of radiation therapy in early stage prostate cancer is to sterilize tumor cells effectively while causing minimal damage to normal cells [Porter AT, 1999]. Radiation can also be introduced into the prostate directly by using interstitial implantation of radioactive isotope seeds. This is referred to as brachytherapy. Brachytherapy has been gaining ground as an alternative to surgery. Traditionally, radiotherapy has not been considered to be as effective as surgery, but brachytherapy has certain advantages. It offers patients a quality of life superior to that of radical prostatectomy or external beam radiotherapy [Future Oncology (a), 1998].

Hormonal therapy

Hormonal therapy is also known as androgen-deprivation therapy, androgen blockade (or ablation), and anti-androgen therapy. Androgens regulate prostate growth of both malignant and benign tissue by an endocrine and paracrine mechanism [Ziada A, 2000]. The importance of androgen deprivation in the management of advanced prostate cancer was demonstrated by Huggins and Hodges in 1941 [Huggins C, 1941]. Since no single agent hormone therapy can completely stop androgen production [Bostwick DG, 1999], combination hormone therapy (CHT) has been explored as alternative approach for prostate cancer [Furr BJA, 1999] and is widely used. CHT combines the drugs to reduce or block the production of testosterone from the testicles and adrenal glands. Androgen ablation remains the primary systemic therapeutic modality for metastatic prostate

cancer. However, in most cases prostate cancer eventually becomes androgen independent, resulting in relapse after a certain period of time [Future Oncology (a), 1998]. Treatment with hormones is a palliative strategy, but not a substitute for other cure-attempting methods [Bostwick DG, 1999].

Cryosurgery

Cryosurgery was first developed in the 1960's [Loening S, 1984]. The procedure is also known as cryoablation or cryotherapy. It uses probes to flash-freeze diseased tissue by using liquid nitrogen (-192°C). Initially, hopes were high that the technique might be valuable in treating cancers located within organs such as the prostate. But cryosurgery did not always fully destroy tumors, and often cancer cells grow back [Bostwick DG, 1999]. For patients with low-grade, low-volume, and low PSA prostate cancer, cryosurgery offers a very acceptable side-effect profile [Blasko JC, 1995]. Cryosurgery is a minimally invasive surgical approach designed for the treatment of prostate cancer [Benoit R, 1999]. But the best cryosurgical technique has not been completely established. It remains an evolving modality [Carroll PR, 2000].

Chemotherapy

Chemotherapy is commonly used in many other cancers, but prostate cancer is an exception. There are no known drug treatments that can cure prostate cancer at any stage [Bostwick DG, 1999]. In most cases, chemotherapy is reserved for patients who have failed hormonal therapy, but advanced prostate cancer usually does not respond well to chemotherapy [Future Oncology (b), 1998]. No standard chemotherapy regimen has been

defined [Sternberg CN, 1999]. Few trials have used chemotherapy in the adjuvant setting combined with hormones. In these trials, patients were pre-treated for a certain period of time with a luteinizing hormone-releasing hormone (LHRH) analogue and flutamide, or LHRH agonist and flutamide, or with cyproterone acetate, or estramustine prior to prostatectomy, or radiation therapy. These new combinations with activity in hormone refractory prostate cancer have been identified, raising the hope that these combinations may prove effective in the adjuvant setting [Chay C, 2001].

1.2. Immunological approaches for the treatment of prostate cancer

Conventional treatments for prostate cancer remain controversial. Even for localized prostate cancer, there are varying opinions regarding their efficacy and side effects [Patel B, 2000]. Prostate cancer is potentially curable by radical surgery or radiotherapy if detected at an early stage. But approximately 50% of newly diagnosed prostate cancer patients have a tumor which has already spread beyond the capsule of the prostate [Hrouda D, 2000]. Further, up to one third of patients treated for prostate cancer relapse [Slovin SF, 1998]. Patients with recurrent or metastatic prostate cancer have few treatment options. Metastatic prostate cancer presents a difficult therapeutic problem for clinicians because no curative treatments are available. Androgen reduction therapy is commonly used to control metastatic growth [Tjoa BA, 2000], but it is not effective for androgen independent metastatic prostate cancer. In most cases, prostate cancer eventually becomes androgen independent. Neoadjuvant hormone therapy has shown some promise by reducing the risk of positive surgical margins and biochemical recurrence in radiation treatment, but has not shown improved overall survival [Patel B,

2000]. Novel therapeutic approaches for prostate cancer certainly need to be explored. Immunotherapy is an attractive novel approach to treat metastatic prostate cancer. In the past, it was believed that prostate cancer was not immunogenic, however this concept has been proven incorrect [Future Oncology (c), 1998]. Prostate cancer usually progresses slowly, allowing several years to pass before the malignancy becomes clinically significant. According to some studies, this growth may take 10 to 15 years. It is therefore a good model to explore immunotherapeutic approaches. New immunotherapeutic approaches for prostate cancer have generated significant interest. Numerous approaches are currently being investigated. These approaches are based on a variety of methodologies with an aim to elicit either humoral or cellular immunity against prostate cancer.

1.2.1. Target antigens for prostate cancer immunotherapy

Several antigens have been discovered and utilized as the target antigens of the corresponding immunotherapy for prostate cancer. Theoretically, ideal target antigens should exhibit certain properties in order to represent ideal candidates for immunotherapy [Saffran DC, 1999]:

1. The antigen should be prostate-specific, expressed at high levels in prostate cancer, and not be expressed in any other tissues or organs;
2. The antigen should be expressed on the cell surface, where it is amenable to recognition by either free or conjugated antibodies;
3. The antigens should be cell surface proteins which play a role in cellular integrity and therefore are desired targets for antibody-mediated inhibition;

4. The antigen needs to be accessible to antigen-presenting cells for processing into MHC class I and II molecules.

Table 1-1 highlights several antigens that are over-expressed in prostate cancer, some of which are currently being evaluated in pre-clinical and clinical studies.

Table 1-1. Target Antigens for Prostate Cancer Immunotherapy

Target Antigen		Function	Immunotherapy Potential
Cell surface proteins	PSMA	Homology to NAALADase	MAb cancer vaccine
	PSCA	Unknown	MAb cancer vaccine
	HER-2/neu	Activated tyrosine kinase receptor	MAb cancer vaccine
Secreted proteins	PSA	Serine protease	MAb conjugate cancer vaccine
	PAP	Acid phosphatase	MAb conjugate cancer vaccine
Intracellular (nuclear) proteins	PAGE	Unknown	Cancer vaccine
	GAGE	Unknown	Cancer vaccine

1.2.1.1. Cell surface antigens

Prostate-specific membrane antigen (PSMA) was originally identified as a result of the generation of specific monoclonal antibodies (MAbs) against membrane preparations of the prostate carcinoma cell line LNCaP [Horoszewicz JS, 1987]. Later studies found that PSMA is a type II integral membrane protein with a short amino-terminal cytoplasmic

domain containing N-acetylated, α-linked acidic dipeptidase (NAALADase) hydrolase activity [Carter RE, 1996]. The presence of a hydrolase domain suggests that PSMA might function by hydrolyzing specific peptides in prostatic fluid or the surrounding environment [Saffran DC, 1999]. Several MAbs have been developed against either intracellular epitope or the extracellular portion of PSMA. Internalization studies of some of these MAbs showed that MAbs could increase PSMA endocytosis in LNCaP cells as well as MAbs themselves. This suggests a potential mechanism to deliver toxic drugs or radioisotopes into prostate tumors [Saffran DC, 1999]. Furthermore, these antibodies recognize PSMA on prostate epithelial cells (normal and BPH) as well as on other organs' epithelia: *e.g.* kidney, colon, breast, bladder, brain *etc.*. This suggests that in addition to using these MAbs to target to prostate cancer epithelial cells, a broader application may be targeting the neovasculature of multiple tissue types.

The immunogenicity of PSMA was evaluated *in vitro*. Dendritic cells (DC) from a prostate cancer patient pulsed with an HLA-A2 specific PSMA peptide, could stimulate autologous T-cell proliferation [Tjoa BA, 1996]. Intact PSMA derived from LNCaP membranes or baculovirus-derived PSMA could also stimulate proliferation of T-cells from either healthy donors or prostate cancer patients [Lodge PA, 1999]. The utility of autologous DC pulsed with PSMA peptides was studied in several clinical trials. Prostate cancer patients with advanced, hormone-resistant disease were administrated autologous DC pulsed with PSMA peptides, and about 30% of the patients showed either a partial or complete response at the end of the studies [Murphy G, 1996; Tjoa BA, 1997]. The combined results of these studies suggest that autologous DC pulsed with PSMA peptide

provided an alternative therapy for advanced prostate cancer, and also should facilitate initiation of trials with other prostate specific antigens [Saffran DC, 1999].

Prostate stem cell antigen (PSCA) was discovered using the LAPC-4 xenograft model named for Los Angeles Prostate Cancer, in an effort to identify genes associated with prostate cancer progression [Reiter RE, 1998; Klein KA, 1997]. Explants from prostate cancer patient tumor sites were implanted into severe combined immunodeficient mice (SCID), in order to expand of small amounts of tissue for further analysis [Klein KA, 1997]. From this model the gene of PSCA was found which encodes a protein composed of 123 amino acids with an amino-terminal signal sequence and a carboxyl-terminal GPI-SCA-2 anchor sequence [Mao M, 1996]. A mouse homolog of PSCA was also identified with 70% identity to the human gene. PSCA mRNA is expressed in normal prostate and placenta, but the levels of mRNA are up-regulated in both xenografts and clinical specimens, and expression is significantly higher than seen in normal prostate [Saffran DC, 1999]. Polyclonal and monoclonal antibodies were generated against human PSCA by either native or denatured PSCA, respectively. These antibodies were utilized to demonstrate the PSCA expression levels on the different cell surfaces [Gu Z, 1999; Reiter RE, 1998]. Currently the function of PSCA is unknown. The facts that a mouse homolog exists and PSCA remains expressed at all stages of disease suggest that it could be a candidate for cancer vaccine for prostate cancer [Saffran DC, 1999].

HER-2/neu, also referred to as erbB2, is an oncogenic protein that is a member of the epidermal growth factor receptor (EGFR) family [Earp HS, 1995]. It is overexpressed in

breast cancer, ductal carcinomas and ovarian cancers. The US Food and Drug Administration (FDA) has approved a monoclonal antibody (Herceptin) to treat HER-2/neu positive tumors in patients with advanced breast cancer [Saffran DC, 1999]. HER-2/neu expression was found in both normal and cancerous prostate epithelial cells, however, conflicting results have been obtained with respect to the level of HER-2/neu overexpression on normal and cancerous prostate epithelial cells [Saffran DC, 1999]. The potential function of HER-2/neu is found in the acquisition of androgen-independence in prostate cancer. Overexpressed HER-2/neu was found on the subline of androgen-independent LAPC (a xenograft model) prostate cancer xenografts. HER-2/neu is also overexpressed in the induction of hormone-independent tumor growth in breast cancer cell lines [Craft N, 1999; Yeu S, 1999]. These studies demonstrated that HER-2/neu may play an important functional role in progression to androgen independence. Thus, it represents a potential avenue for immunotherapy. Disis [Disis ML, 1997] reviewed that in a variety of model systems, HER2/neu has been shown to induce specific T-cell immunity, suggesting its potential as a cancer vaccine candidate.

1.2.1.2. Secreted antigens

Prostatic acid phosphatase (PAP) is a prostate-specific secreted protein, expressed in normal or cancerous epithelial cells, but not in any other tissues investigated [Lam KW, 1989; Solin T, 1990; Sinha AA, 1998]. Circulating PAP levels in the serum of prostate cancer patients increase progressively with the stage of disease [Jacobs EL, 1991]. Immunogenicity of PAP has been evaluated *in vivo* and *in vitro. In vivo,* PAP-specific antibody (Ab) response or CTL response could be induced by either PAP immunization

of rats or by vaccinia virus expressing PAP. *In vitro,* PAP peptide pulsed DC could stimulate production of CTLs which have the ability to lyse PAP peptide-pulsed HLA-A2+ target cells as well as PAP-expressing LNCaP cells. Abs to PAP have been investigated for their ability to target drugs to prostate cancer cells and tissues [Sinha AA, 1998; Deguchi T, 1986 and 1987]. *In vitro* and *in vivo* studies of drug conjugated Abs showed that they certainly targeted LNCaP cells and tumors, and inhibited the growth of either LNCaP cells or tumors [Sinha AA, 1990]. Because of its immunogenicity, specificity and overexpression in all stages of prostate cancer, PAP is a potential prostate cancer vaccine candidate.

Prostate specific antigen (PSA) is a well studied and utilized secreted antigen expressed by prostate cells. It has been studied more extensively than other prostate tumor antigens. Biochemically, it is characterized as a single chain glycoprotein that contains 93% amino acid and 7% carbohydrate. Its molecular weight is 34KD. Functionally, PSA is a kallikrein-like serine protease that is produced exclusively by the epithelial cells of the prostate gland [Buck AC, 1995]. Normally, PSA is found in high concentrations in seminal plasma and in extremely low concentration in the circulation. However, once tumor has developed in the prostate gland, the serum level of PSA will considerably increase. This makes it a useful serum marker for diagnosis of prostate cancer [Oesterling JE, 1991; Lee CT, 1995]. In the circulation, PSA exists as native molecule but predominantly in complexed forms. PSA is found bound either to α-2-macroglobulin (AMG) or to α-1-antichymotrypsin (ACT), two of the major serine protease inhibitors in blood [Vessella RL (a), (b), 1993]. In clinical medicine, PSA is the tumor marker that has

been approved in 1986 by FDA for mass screening to diagnose early prostate cancer. Since then, the level of PSA and the ratio of detectable PSA/PSA-ACT in the circulation, have been widely used for population screening, diagnosis and monitoring of patients with prostate cancer, and PSA is considered as the best prostate tumor marker so far.

The physiology and pathobiology of PSA are still not well understood. PSA may act as a tumor suppressor, a negative regulator of cell growth, and as an apoptosis-inducing molecule. But some other reports suggest that PSA may, through its chymotrypsin-like activity, promote tumor progression and metastasis. Because PSA is just one member of the human kallikrein gene family, it is possible that its biological functions are related to the activities of other related kallikreins. Physiological functions of PSA and other kallikreins need to be elucidated [Diamandis EP, 2000].

PSA has been used increasingly as it is the key marker of a therapeutic response. As a candidate of immunological therapy of prostate cancer, PSA matches most of the categories of ideal candidates listed earlier of this section. The fact that PSA is found in very high concentration inside the tumor and metastatic sites makes it a good target for immune intervention. The immunogenicity of PSA has been positively addressed. The ability of PSA to induce specific T-cell mediated immune responses has been evaluated in both murine and human *in vitro* and *in vivo* model systems. Immune response specific for PSA could be induced by PSA, a PSA transfected syngeneic cell line and a PSA overexpressing cell line [Wei C, 1996; 1997]. Cytotoxic T lymphocytes (CTL) specific for PSA have been generated *in vitro* by stimulating peripheral blood lymphocytes (PBL)

from normal HLA-A2 individuals with PSA peptides targeted to the MHC class I allele HLA-A2 [Xue BH, 1997]. Similarly, *in vivo* PSA specific CTL could be generated from PBL of normal subjects or prostate cancer patients using PSA peptides. The PSA-specific CTL had the ability to lyse either peptide-pulsed target cells or the HLA-A2 positive prostate carcinoma cell line, LNCaP [Correale P, 1997, 1998]. These studies demonstrate that a significant human CTL immune response can be generated against PSA [Saffran DC, 1999]. Thus, the immunogenicity of PSA was addressed.

A murine homolog of human PSA has not yet been identified [Saffran DC, 1999]. Recently, as a cancer vaccine, PSA has been evaluated in prostate cancer patients in clinical trials. PROSTVAC, a recombinant vaccinia virus expressing PSA, has been evaluated in prostate cancer patients after radical surgery [Sanda MG, 1999]. This vaccine showed long-lived PSA T-cell responses in a monkey preclinical study. However, in humans, it didn't show significant results. Only one patient out of six showed a clinical response of undetectable serum PSA. Another vaccine, OncoVax-P, baculovirus produced human PSA protein mixed with liposomes and the adjuvant lipid A, showed that a significant proportion of patients had a successful immnunization response against PSA by measuring the circulating antibodies and delayed type hypersensitivity (DTH) response, with no serious side effects [Harris DT, 1999]. Both studies demonstrated the potential utility of PSA as a cancer vaccine candidate for prostate cancer.

Also, Abs against PSA have been investigated for their ability to target drugs and to inhibit growth of prostate cancer cells and tissues. Various studies demonstrated that

antibodies can localize PSA to prostate epithelial cells and effectively deliver therapeutic drugs [Saffran DC, 1999]. Anti-PSA MAb, which was developed in our laboratory, is a well characterized MAb. Previous work has demonstrated that it has high binding affinity to human PSA. A delay in the progression of PSA expressing tumors was observed in mice after i.v. administration anti-PSA MAb as a therapeutic agent.

Two novel serine proteases, prostase and TMPRSS2, which are similar to PSA, have been identified. Both are androgen-regulated and overexpressed in cancerous prostate gland [Nelson PS, 1999; Yousef GM, 1999; Lin B, 1999]. Antibody therapy or vaccine approaches might be considered for these targets. Further expression analysis of these two proteases on a larger panel of prostate cancer specimens will be required to confirm the relevance of these proteins as target antigens for immunotherapy [Saffran DC, 1999].

1.2.1.3. Intracellular antigens

Several nuclear proteins were identified, and named as PAGEs (prostate-associated gene) and GAGE. They were homologous to MAGE (melanoma-associated gene) family, and expressed in prostate, testicular and female reproductive tissues. The expression of these genes needs to be examined in a larger panel of clinical samples [Brinkmann U, 1998], but nonetheless PAGE-4 is currently being evaluated as a target for vaccine therapy of prostate cancer [Brinkmann U, 1999].

1.2.2. Gene therapy

Cancer gene therapy refers to treatment strategies where gene transfer methods are used to generate immune responses against cancer [Ribas A, 2000]. Gene therapy involves the

transfer of DNA into cells to replace or to affect the expression of the cells' native (endogenous) genes. This transfer may occur *ex vivo* (while the cell is outside the body) and then returned to the body, or the gene may be introduced into the cell *in vivo* (while the cell remains in the body in its natural microenvironment). To transfer DNA into a cell with sufficient efficiency, a DNA transporter or "vector" is generally required. Methods used to transfer genes into cells are broadly categorized as either nonviral based or viral based [Gingrich JR, 2001] and have recently been summarized in several papers [Vile RG, 2000; Monahan PE, 2000; Li S, 2000]. In general, viral gene therapy techniques have tended be more popular due to higher rates of gene transfer efficiency in recent times. The most commonly used viral vectors have been adenoviruses. They have the advantage of transferring genes into both dividing and nondividing cells. Other examples of viral vectors include retroviruses and adeno-associated viruses, which are capable of integration into the DNA of dividing cells. One commonly used nonviral delivery system is liposomes. Encapsulated DNA in liposomes or DNA-liposome complexes had significantly higher transformation efficiency than naked DNA alone [Felgner PL, 1987]. Liposomes have the advantage over viral vectors in that they are safer, with diminished risk of immunological and pathological complications [Patel B, 2000].

Prostate cancer is particularly suited to gene therapy for a number of reasons:

1. Prostate cancer is quite common and for the patients diagnosed with locally advanced disease there is no cure currently available;

2. The prostate gland does not serve any life-sustaining function and therefore complete ablation of benign, premalignant, and cancerous prostate tissue could be undertaken;
3. The prostate is accessible by transurethral, thansperineal, and transrectal approaches for administration of gene therapy;
4. The prostate may be monitored by DRE, transrectal ultrasound, endorectal coil magnetic resonance imaging, and/or by serum PSA;
5. The prostate gland produces high levels of several characterized proteins including PSA, whose promoters and other enhancers may be incorporated into vectors to direct prostate-specific expression of therapeutic genes.

However, prostate cancer also poses some unique challenges for gene therapy. Less than 5% of cells are actively dividing at any one time within a tumor, and the mean doubling time of prostate cancer has been estimated to be greater than 150 days [Berges RR, 1993]. Because of this low proliferative index, vectors that have high gene transfer rates and that are independent of cell division will be required. Another fact is that prostate cancer is very heterogeneous, not only from individual to individual, but also within the same individual. Thus, appropriate gene therapy for one individual may not be effective to treat another individual [Gingrich JR, 2001].

Numerous gene therapy strategies are currently being employed for the treatment of prostate cancer [Steiner MS, 2000]. These approaches can be categorized as those designed by one of the following:

1. Immunotherapy to enhance the host immune system antitumor response. By using *ex vivo* gene transferred vaccine; or *in vivo* intratumoral injection of vectors containing cytokine genes;
2. Corrective gene therapy to increase or abrogate specific aberrant gene expression that is important for normal growth regulation. Tumor suppressor genes and oncogenes often are objectives;
3. Suicide gene therapy by introducing toxic or cell lytic genes results in cell destruction;
4. Combination therapy: one or more of these strategies with conventional treatments.

Currently 32 gene therapy trials have been approved by the National Institutes of Health of the United States for the treatment of prostate cancer. Thus far, prostate gene therapy appears to be safe and well tolerated. These studies are encouraging for application of gene therapy for human prostate cancer. Gingrich [Gingrich JR, 2001] reviewed recently in detail current gene therapy clinical trials for prostate cancer in *Current Oncology* **3**: 438-447, 2001.

Both conventional treatments and novel immunotherapy approaches for prostate cancer are being investigated and considerable progress has been evidenced. Nevertheless, future studies including novel prostate cancer specific antigen discovery, optimization of vaccine delivery and combination conventional and immunotherapy, are suggested for further investigation to yield more effective protocols for advanced prostate cancer.

1.2.3. Dendritic cell based immunotherapy

1.2.3.1. Generalities of dendritic cells

Dendritic cells (DC) are a group of leukocytes that derive from hematopoietic progenitors [Clark GJ, 2000]. DC are distributed in most tissues (excluding brain) [Bell D, 1999], and in particular, in tissues that interface with the external environment, where microorganisms can enter [Rescigno M, 1999]. Their functions in the immune system are believed to be foreign antigen processing and presenting to initiate certain immune responses against the foreign antigen.

Following the discovery of DC culture techniques in early 1990s, the knowledge of physiology and the mechanisms of antigen presentation have progressed considerably. DC comprise three distinct subsets, including two within the myeloid lineage, Langerhans cells and interstitial DC, and one within the lymphoid lineage, the so-called lymphoid DC subset. There are three stages of development, *i.e.*, precursor DC patrolling through blood and lymphatics, immature DC residing within virtually every tissue in ambush to capture pathogens, and mature DC residing temporarily within secondary lymphoid organs. DC also appear to play an important role in the induction of immunological tolerance. In particular, thymic DC present endogenous self-peptides to newly generated thymocytes, thereby allowing the deletion of self-reactive T cells [Bell D, 1999].

Distributed as sentinels throughout the body, precursor DC are poised to capture antigen, and migrate to draining lymphoid organs. After a process of antigen degradation, DC

present the processed antigen to rare, antigen-specific T cells to initiate their expansion and, thereby initiate clonal immunity maturation [Figure 1-1, Palucka K, 1999]. Current studies consider that DC is an essential link between innate and adaptive immunity [Palucka, 1999]. Studies have revealed that DC support the generation of not only lymphokine-secreting helper T cells, but also cytotoxic T lymphocytes (CTLs), which subsequently migrate to the site of initial injury to eliminate virally infected cells or tumor cells. The capacity of activating not only memory, but also naïve T cells is a property not shared by other antigen-presenting cells (APCs). Hence, DC are so-called professional APCs [Bell D, 1999]. This unique character of DC has obtained more and more attention of researchers. There is a growing list of research on the mechanism of DC antigen uptake and presentation.

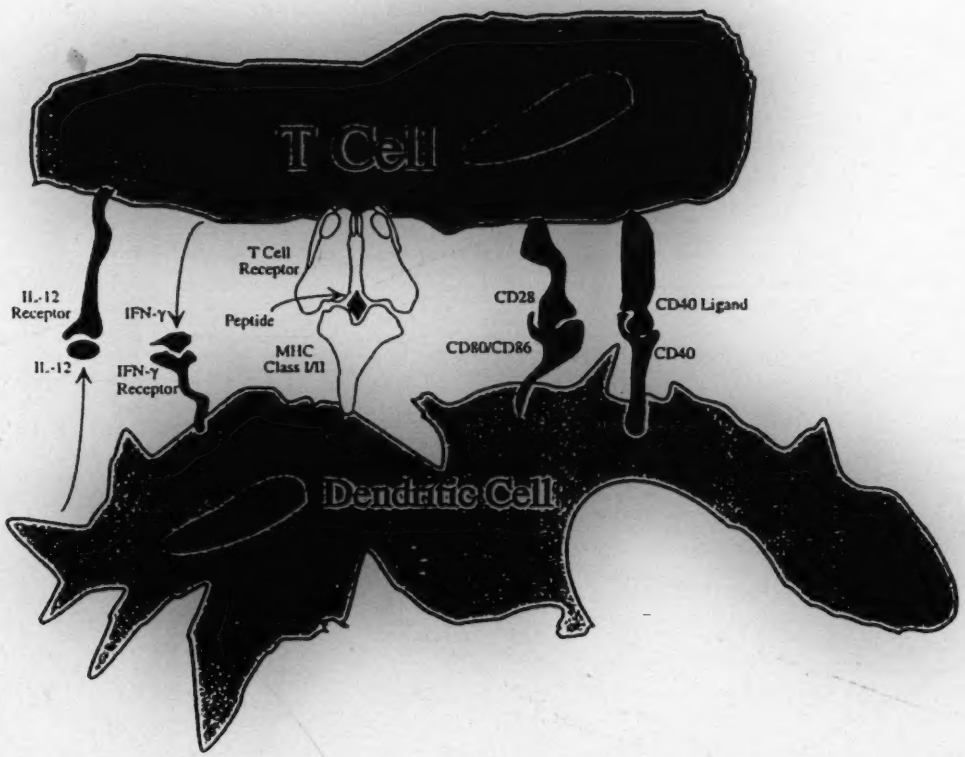


Figure 1-1. Interactions between a T cell and a dendritic cell involved in T cell activation. T-cell receptor recognition of peptide antigen/MHC product is shown. In addition, binding of costimulatory molecules and their ligands, and certain cytokines and their receptors are shown.

Cited from Benjamin A. Tjoa, *et al.*. (1999) Vaccine therapy for prostate cancer. *Urologic Clinics of North America*, 26 (2).

1.2.3.2. Antigen uptake by dendritic cells

Immature DC can efficiently internalize a diverse array of antigens for processing, as a consequence of high endocytic activity levels. Antigen uptake by DC can occur via three distinct mechanisms:

1. Macropinocytosis: Macropinocytosis is a cytoskeleton-dependent type fluid-phase endocytosis; it is constitutive and enables a single cell to take up a very large volume of fluid mediated by membrane ruffling and the formation of large vesicles.

2. Receptor-mediated endocytosis: A number of receptors are involved in endocytosis. These include Fc receptors, the MHC molecule, CD14, DEC-205 and mannose receptors and the new family of "danger" receptors called TLR (toll-like receptor). DC express several receptors for the Fc portion of immunoglobulin (Ig), which mediate internalization of antigen-Ig complexes and promote efficient major histocompatibility complex (MHC) class I and II presentation [Regnault A, 1999]. The Mac-1 molecule is the CR3 complement receptor used for the phagocytosis of complement-coated bacteria, but it can respond to chemoattractants to mediate adhesion and chemotaxis. The mannose receptor is a 175 KDa C-type lectin (which homologous with DEC-205 receptor), which is expressed predominantly on DC and macrophages and involved in the internalization and presentation of mannosylated proteins. TLR is a family of proteins that have been described as key players in triggering innate defenses against bacterial and fungal invaders.

3. Engulfment of apoptotic bodies: DC can capture and engulf apoptotic cells, but not necrotic cells, preferentially through the vitronectin receptors.

1.2.3.3. Antigen presentation by dendritic cells

MHC class II pathway

MHC class II loading is critical to $CD4^+$ T cells. Immature DC constantly accumulate MHC class II molecules in MHC class II-rich compartments (MIICs) ready for loading exogenous soluble antigen peptides. MHC class II molecules in MIICs are targeted by invariant chain (Ii chain) from endoplasmic reticulum (ER). HLA-DM molecules in MIICs will remove the Ii-derived class II-associated invariant chain peptide (CLIP), and form MHC and peptide stable complexes. During their maturation, DC undergo major changes in phenotype and function, and a large number of surface MHC class II molecules will be expressed to fulfill the variety of antigens' presentation strategies [Bell D, 1999; Banchereau J, 2000]. Figure 1-2 presents antigen presentation by DC through the MHC class II pathway.

MHC class I pathway

MHC class I loading is critical for $CD8^+$ T cells. Traditionally, cytosolic proteins are degraded into short peptides and are loaded on newly synthesized MHC class I molecules within ER *via* ATP-dependent TAP 1 / 2 transmembrane transporters and are trimmed into 8-10 amino acid residues which accommodate the MHC class I-binding groove. Figure 1-3 presents antigen presentation by DC through MHC class I pathway.

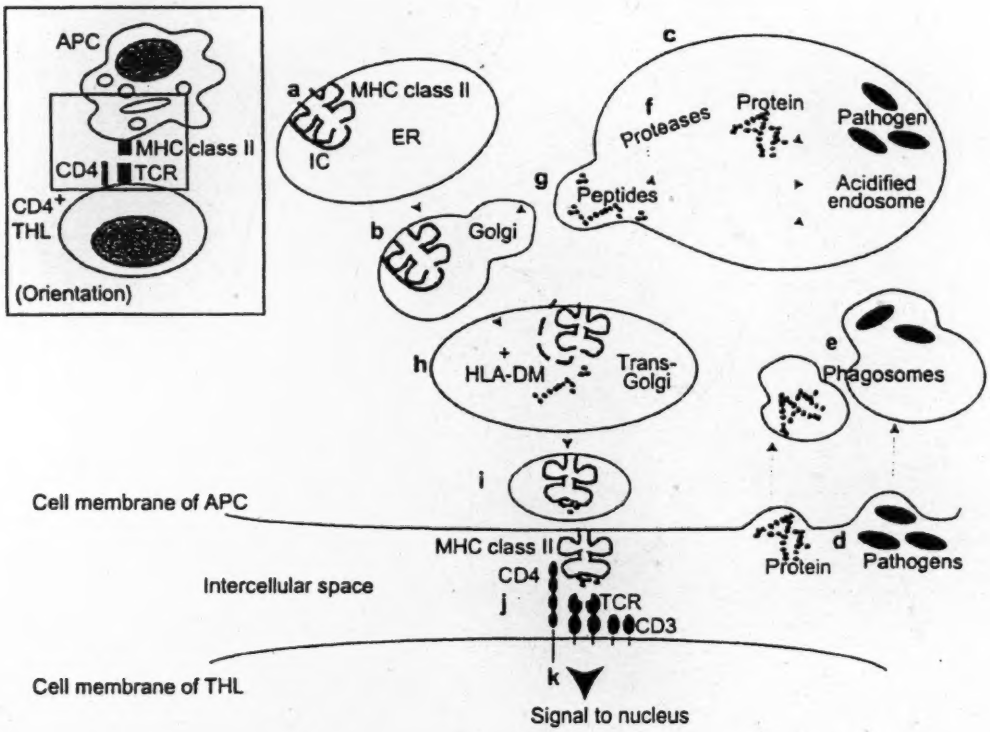


Figure 1-2. Degradation and transport of antigens that bind major histocompatibility complex (MHC) class II molecules. (a) In an antigen-presenting cell (APC), newly synthesized MHC class II molecules bind the invariant chain (IC), which prevents binding of peptides that are present in the endoplasmic reticulum (ER). (b) The IC allows transport of MHC class II molecules form the ER into the Golgi apparatus to acidified endosomes. (c) Endosomes contain peptides that are derived from either resident pathogens (e.g. bacteria) or (d) engulfed extracellular proteins (or pathogens) (e) in the phagosomes. (f) Proteases within the endosome degrade proteins into peptides. (g) The endosome fuses with the Golgi to form the trans-Golgi. (h) Here, the IC is cleaved and released from the MHC class II molecule. This allows the binding of peptides within the endosome to the peptide-binding cleft of the MHC class II molecules. An MHC-class-II-like molecule (HLA-DM) binds to MHC class II molecules to facilitate the release of the IC (not shown). (i) The MHC class II-peptide complex is then transported to the cell surface of the APC for (j) recognition by the T-cell receptor (TCR) of ($CD4^+$, $CD3^+$) T-helper lymphocytes (THLs) and (k) intracellular signaling for activation.

First published in: Man, S. (1998) Human cellular immune responses against human papillomaviruses in cervical neoplasia. *Exp. Rev. Mol. Med.* Txt001 smc, 3 July 1998.

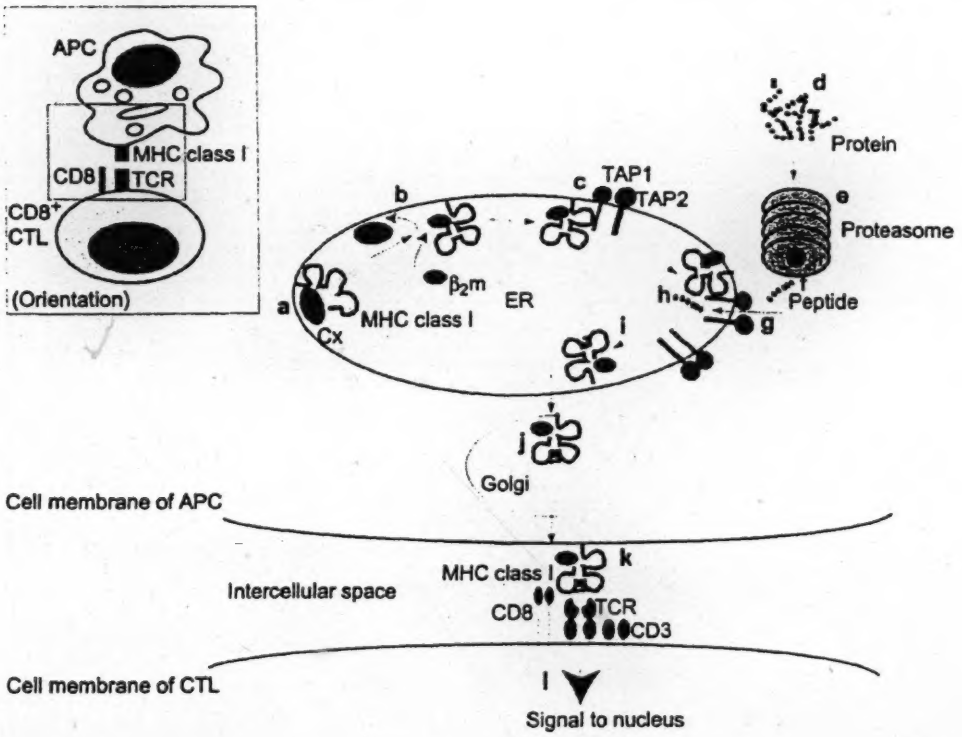


Figure 1-3. Degradation and transport of antigens that bind major histo-compatibility complex (MHC) class I molecules. (a) In an antigen-presenting cell (APC), newly synthesized MHC class I molecules bind to calneix (Cx), which retains them in a partially folded state in the endoplasmic reticulum (ER). (b) Binding of MHC class I molecules to β_2 microglobulin (β_2m) displaces Cx and allows binding of chaperonin proteins (calreticulin and tapasin; not shown). (c) The MHC class I-β_2m complex binds to the TAP complex (TAP1-TAP2), which awaits the delivery of peptides. (d) Peptides (e.g. from antigens) are formed from the degradation of cytosolic proteins ('self'-, pathogen-and tumor–derived proteins in the cytoplasm). (e) These are degraded by proteasomes into (f) short peptides. (g) Peptides are transported into the ER by the TAPs, where they meet the MHC class I-β_2m complex (h). This peptide binding in the antigenic groove of the MHC stablises the structure of the MHC class I molecule and (i) releases the TAP complex. (j) The fully folded MHC class I molecule with its peptide is transported to the cell surface via the Golgi apparatus. (k) Recognition of the MHC class I-peptide complex by the T-cell receptor (TCR) of an antigen-specific ($CD8^+$, $CD3^+$) cytotoxic MHC-class-I restricted T cell; this required co-stimulation to occur (not shown).

First published in: Man, S. (1998) Human cellular immune responses against human papillomaviruses in cervical meoplasia. *Exp. Rev. Mol. Med.* Txt001 smc, 3 July 1998.

The nature of the pathway of MHC class I peptide loading has long been known to exclude the possibility that non-endogenously produced proteins could be presented on these MHC class I molecules. However, more and more studies show that DC can actually capture exogenous antigens and load them on MHC class I molecules and induce a clonal CTL response [Rescigno M, 1999]. This mechanism was described as "cross-priming" [Norbury CC, 1997]. Basically, two routes for the exogenous MHC class I pathway have been described, a TAP-independent pathway in which antigen is most likely hydrolyzed in endosomes, and a TAP-dependent pathway, which is a phagosome-to-cytosol pathway [Banchereau J, 2000]. Most recently, Fcγ-mediated capture and presentation of immune complexes and exosomes derived either from tumor cells or from tumor-peptide-pulsed DC were demonstrated as another possible pathway permitting access to DC MHC class I presentation.

1.2.3.4. Mannose mediated antigen uptake and presentation by dendritic cells

Biochemically, mannose receptor contains multiple carbohydrate-binding domains [Stahl PD, 1990; Drickamer K, 1993]. It is involved in the internalization of a variety of sugar-containing proteins and has been shown to mediate phagocytosis of various microorganisms which expose mannosylated glycoproteins [Stahl PD, 1990; Pontow SE, 1992; Ezekwitz RA, 1991]. Engering AJ and colleagues [Engering AJ (b), 1997] have studied mannose receptor functions in human DC. They analyzed mannose receptor-mediated antigen uptake as well as the subcellular distribution of the mannose receptor in DC. The results showed that mannose receptor mediated uptake and presentation to T cells is 100-fold more efficient, compared to antigens internalized *via* the fluid phase. In addition, the mannose receptor was localized in vesicular structures distinct from MHC

class II compartments, suggesting that the mannose receptor functions as a reusable antigen receptor for concentration of predominantly non-self antigens for processing and presentation. It is evident from the results that mannose receptor functions as a high capacity and broad specificity antigen receptor in human DC. This process is totally different from Fc receptor capture antigen-antibody complex. Fc receptors allow APCs to rapidly capture antigen-antibody complexes. Fc receptor-ligand (antigen) complexes are internalized by APCs and the antigen targeted to intracellular compartments containing MHC II molecules. The antigen is degraded in a similar way to membrane Ig (mIg) receptors clonally distributed on B lymphocytes, which function in internalization of a single type of antigen. Both Fc receptors and mIg are available for a single round of uptake only [Engering AJ (a), 1997; Lanzavecchia A, 1987; West MA, 1994; Bonnerot C, 1995]. The finding that the mannose receptor was not localized in MHC II class II compartments by Engering and colleagues [Engering AJ (a), 1997] indicates that the mannose receptor-containing compartments are involved in antigen capture only. Agenes MC and colleagues [Agnes MC, 1997] observed that targeting of mannosylated antigens and peptide to the mannose receptor pathway resulted in increased potency (200- to 10,000-fold) to stimulate HLA class II-restricted peptide-specific T cells. They also found [Agnes MC, 1998] the 100-1,000-fold lower concentration of *bis*-mannosylated APL uptake by DC *in vitro* was sufficient for complete blocking of the proliferative T cell response against the agonist peptide compared to the non-mannosylated APL. Moreover, a strong increase in the efficiency of presentation of APL was also observed when macrophages and PBMCs (mannose receptor positive) were used as APCs. Apostolopoulos and colleagues characterized the oxidized mannan (mannopolysaccharide

or polymerized mannose) which contains aldehyde group conjugated MUC-1 fusion protein, which targets the MHC class I antigen-presentation pathway [Apostolopoulos V (a), 1995; 1996]. They [Apostolopoulos V (b), 1995] have found that oxidative mannan conjugation of MUC-1 can be internalized and presented 1,000 times more efficiently by MHC class I than reductive mannan conjugation in animal experiments. The oxidative product and *not* the reductive product could initiate a CTL response specific to antigen MUC-1 [Apostolopoulos V, 1996]. They demonstrated that the mechanism of mannan conjugated antigen capture and presentation is mannose receptor mediated and TAP dependent [Apostolopoulos V, 2000].

1.2.3.5. Dendritic cell-based immunotherapy for prostate cancer

Human tumor immunotherapy has met with only limited success. However, recent insights into the role of DC as the pivotal antigen-presenting cells that initiate immune responses may provide the basis for generating more effective antitumor immune responses. Since DCs initiate not only effector T cells but also naïve T cells, extensive studies on the induction of both humoral and cellular anti-tumor immune responses have been documented. Most of these experiments have involved *in vitro* isolation of DC, followed by loading of the DC with tumor antigen and injection of the antigen-bearing DC into syngeneic animals as a cancer vaccine. In *in vivo* tumor rejection studies in mice, DC pulsed with tumor associated antigens were administered. A 50% survival rate was observed in tumor-bearing mice receiving splenic DC and 0% survival in mice not receiving DC [Knight SC, 1985]. Also, successful prevention of tumor growth *in vivo* was demonstrated by administration of DC pulsed with various antigens, including

autologous tumor fragments, idiotype IgM in a B cell tumor system, and OVA peptides derived from an OVA-transfected tumor cell line. Moreover, dendritic cell-based clinical trials with several types of cancers including prostate cancer have been tested with some success. Tjoa and colleagues have been developing a prostate cancer vaccine using autologous DC as a vehicle which is pulsed with HLA-A2 PSMA peptides present to T cells. This vaccine is being used in clinical trials. The safety and efficacy of administration of autologous DC/PSMA peptides were assessed. In most cases neither significant acute, nor chronic toxicity was observed in all doses of test substances and an increased cellular response was observed in some of the patients [reviewed by Tjoa BA, 2000].

Although the results to date of the various DC trials are equivocal, the procedures used for isolating and "arming" DC are tedious and not yet broadly applicable to clinical practice. Fong [Fong L, 2000] suggested that the source of DC for use in clinical trials and the choice of tumor antigen with which to "arm" the DC are almost certainly going to have a profound influence on clinical outcomes. Combinations of antigens should be used to reduce the risk of generation of antigen-loss variants that evade the immune response [Fong L, 2000]. Antigen delivery also remains to be optimized. Nevertheless, at this stage, the results from attempts to utilize DC for cancer immunotherapy are encouraging. Further integration of fundamental tumor immunology research with well-designed clinical trials of the DC-based approach promises to generate effective anti-tumor immunity against prostate cancer.

1.3. Specific aim

T cells, in particular CD8+ cytotoxic T lymphocytes, are considered to be the principal mediators of anti-tumor immunity. The pivotal role of DC in the induction of cellular immune responses has focused attention on DC as a powerful tool for eliciting anti-tumor immunity. Mannose receptors expressed on DC are involved in processing a variety of antigens and DC will (probably) have the ability to elicit naïve T cell response including CTL immunity.

Preliminary studies in our laboratory demonstrated that an anti-PSA MAb has high binding affinity with human PSA and can induce a specific immune response against PSA. A delay in the progression of PSA expressing tumors was observed in mice after immunization of the animals with the anti-PSA MAb [Leveugle B, 1998]. The formation of anti-PSA MAb and PSA immune complex in the circulation was responsible for the biological effect. APCs will internalize the immune complexes through the Fc receptors on the cell surface and the PSA will be degraded into PSA-peptide. Degraded PSA-peptide will be presented by MHC molecules on the APC cell surface ready to react with PSA specific T-cells, and an immune response specific to PSA will be induced. We believe that induction of a stronger immune response is dependent on more anti-PSA MAb/PSA immune complexes targeting antigen presenting cells.

The studies in this thesis were founded on a literature survey and our preliminary studies on an immunotherapeutic approach for prostate cancer. The studies will focus on utilizing DCs to elicit more efficient anti-PSA immunity against prostate cancer. Two routes will

be studied. One is to conjugate anti-PSA MAb with anti-DC MAb in order to obtain a bispecific agent with PSA binding activity as well as DC binding activity. The other is to mannosylate anti-PSA MAb in order to get presented by DC through mannose receptors on DC. It was hypothesized that modified anti-PSA MAb with DC binding activity would target DC more efficiently than non-modified anti-PSA MAbs. A stronger anti-PSA immune response induction would be anticipated.

Strategies for this study:

1. To modify anti-PSA MAb and PSA, by:

i.) Conjugation of anti-PSA MAb with anti-DC MAbs. Chemically conjugate anti-PSA MAb with three anti-DC MAbs (N418, 33D1 and DEC-205) by using the protein cross linker, glutaraldehyde, to generate bispecific antibody conjugates with specific binding activity to PSA as well as to DC. The conditions of the conjugation method and the evaluation of binding activities of bispecific antibodies to PSA and DC were the subjects of the studies.

ii.) Mannosylation of anti-PSA MAb or PSA. Chemically conjugate anti-PSA MAb with mannose residues. Ideally, mannosylated anti-PSA MAb would maintain similar PSA binding activity compared to anti-PSA MAb; it would have the ability to target DC more efficiently. Considering these two major characteristics of mannosylated anti-PSA MAb, the best conjugation method would be determined by evaluation of modified anti-PSA MAb under different mannosylation conditions. In addition, the capacity of mannosylated anti-PSA MAb targeting to DC was evaluated by utilizing a dendritic cell line *in vitro*.

iii.) Mannan conjugation of PSA. Modifying PSA by conjugating with oxidized mannan was another strategy targeting to DC with the hope of eliciting a specific anti-PSA immune response. The capacity of mannan conjugated PSA targeting to DC would be evaluated.

2. To study the induction of the immune response by modified anti-PSA MAb and PSA, both *in vivo* and *in vitro*. We propose to use a dendritic cell line to study DC uptake of modified anti-PSA MAb and PSA *in vitro*. The radioisotope ^{125}I was recommended as a tracer to track the existence of modified anti-PSA MAb and PSA taken up by DC. Induction of the PSA specific immune response by modified anti-PSA MAb and PSA would be studied both *in vivo* and *in vitro*.

CHAPTER 2

Results and Discussion

2.1. Monoclonal antibody production

Methods for monoclonal antibody (MAb) production are currently well established. To obtain a sufficient amount of antibodies, desired active hybrids can either be large-scale hybridoma cultured or grown in animals [Howard GC, 2001]. Typical yield range for cell culture supernatant is from 5 to 50μg/mL, depending on the individual clones and the cell density in the culture. Ascites production in mice provides a relative economical and fast method for antibody production. The product concentration level of MAb in ascites is from 2 to 10mg/mL. The ascites containing MAbs grown in the animal may contain normal immunoglobulins (Igs, non-specific antibodies). This might limit the ultimate purity and specificity of the final product, but usually it is not a major problem [Goding JW, 1996].

There are many methods available for antibody purification, such as: precipitation with saturated ammonium sulfate, which is one of the oldest and most useful methods; precipitation at low ionic strength; ion exchange chromatography; gel filtration and affinity chromatography. Affinity chromatography is usually the choice for purifying MAbs from culture supernatants [Goding JW, 1996]. It is a method of separation that is based on the irreversible immobilization of a component in a system on a solid-phase matrix. The subsequent binding and elution of a complementary ligand are obtained in solution. Compared to other methods, affinity chromatography is ideally suited to purify monoclonal antibodies from culture fluid, which has relative low concentration (5-

50μg/mL) of the antibodies. Generally, a better way to purify monoclonal antibodies is to bind to and elute from Staphylococcal protein A or Streptococcal protein G. Protein A and protein G have different binding capacities to different classes of antibody species [Goding JW, 1996]. Therefore, for a better purification efficiency the species of the hybrids and the class of the antibody must be known in order to decide whether protein A or protein G chromatography should be applied.

In this research project, four different hybrid MAb productions were studied in large-scale cell culture, as well as in SCID beige mice. Large amounts of MAbs were purified by using protein G chromatography, which showed a better efficiency than protein A chromatography for purifying IgG from mouse, rat or hamster.

2.1.1. Hybridoma cell lines

DEC-205 hybridoma

DEC-205 (syn. NLDC-145, from ATCC) is a rat-mouse hybridoma cell line, obtained from the fusion of the mouse Sp2/0-Ag14 myeloma with the B-cells of a rat. The rat was immunized with mouse lymph node stroma. This hybridoma clone secretes IgG2a MAbs which react with the DEC-205 antigen expressed at high levels on mouse dendritic cells (DC) and thymic cortical epithelial cells. DEC-205 antigen is an integral membrane glycoprotein homologous to the mannose receptor (Bell D, 1999) with an apparent mass of 205 KD. DEC-205 has been identified as a putative antigen-uptake receptor that efficiently internalizes antigens and leads to presentation to T-cells [ATCC product information sheet].

33D1 hybridoma

33D1 (form ATCC) is a rat-mouse hybridoma cell line, obtained from the fusion of mouse P3X63Ag8-UI myeloma cells and B-cells of a rat immunized with mouse spleen and lymph node DC. This hybridoma clone secretes IgG2b MAbs directly against antigen only expressed by mouse DC. The antigen recognized by the 33D1 MAb does not appear to be immune response associated antigen or Ia antigen [Nussenzweig MC, 1982; ATCC product information sheet].

N418 hybridoma

N418 (from ATCC) is hamster-mouse hybridoma cell line, obtained from the fusion of mouse Sp2/0 myeloma cells and B cells of an Armenian hamster immunized with mouse DC. This hybrid clone secretes IgG MAbs, which precipitate leukocyte integrin (CD11c/CD18 heterodimer) expressed on mouse DC. The N418 epitope is detected only on mouse DC and is not present on peritoneal macrophages. The N418 epitope may be the mouse equivalent to human CD11c [Metlay JP. *et al.*, 1990; ATCC product information sheet].

Anti-PSA MAb hybridoma

Anti-PSA MAb hybridoma cell line has been previously generated in our laboratory by the fusion of mouse Sp2/0 myeloma cells with mouse B-cells from a mouse immunized with human prostate specific antigen (PSA). This hybridoma clone secretes IgG_1 MAb,

which recognizes human PSA. This MAb recognizes the epitope that is comprised among residues 139-164 of PSA.

2.1.2. Monoclonal antibody production

The production of anti-DC MAbs was performed by large-scale hybridoma cell culture and ascites formation. The different clones of anti-DC MAbs were cultured in standard medium, hybridoma serum free medium (SFM), and ultradoma medium (UD), respectively. Table 2-1 shows MAb concentrations in cell culture media and in ascites fluid, as well as the yields of the purification by protein G chromatography.

The concentrations of anti-DC MAbs varied from each cell culture supernatant. In SFM supernatant (before concentration), it was at the low end (less than 18μg/mL) of the typical range (5-50μg/mL) of culture supernatant. The anti-DC MAb's concentration was even lower in the UD supernatant (less than 10μg/mL). This implied that in order to obtain higher quantity of anti-DC MAbs, SFM is superior to UD hybridoma culture with respect to product concentration.

Anti-DC hybridoma clones (DEC-205, 33D1, and N418) are of non-murine in origin. To avoid generation of anti-anti-DC Abs immune response in normal mice, ascites production of anti-DC MAb hybridomas was studied in nonimmunocompetent mice. Mice with severe combined immunodeficiency disease (SCID) were used for generation of ascites fluid. SCID beige mice do not have the ability to develop mature T and B cells, and consequently are severely immunologically compromised. The amounts of ascites

fluid obtained from SCID beige mice varied from one clone to another. Reasonable amounts of ascites fluid were generated from the N418 clone, about 5mL per mouse, but with low MAb concentration (25.6μg/mL). The 33D1 and DEC-205 hybridomas failed to generate sufficient amounts of ascites fluid; the fluid was less than 1mL per mouse. The concentration of 33D1 MAb was also low in ascites (17.6μg/mL). Although DEC-205 hybrid did not generate much ascites, its MAb concentration (3130μg/mL) was much higher than those of N418 and 33D1 hybrid ascites. Detailed results are shown in Table 2-1.

Table 2-1 Purified Monoclonal Antibody Products

MAbs	Antibody concentration starting materials (μg/mL)		**Purified MAbs**	**Yield (%)**
N418 MAb	SFM	5.5	5mg	66
	UD	0.2	0.28mg	93
	Ascites	25.6	0.52mg	100
33D1 MAb	SFM	18.0	14mg	54
	Ascites	17.6		
DEC-205 MAb	SFM	10.4	17mg	93
	UD	8.0	20mg	100
	Ascites	3130	20mg	60
Anti-PSA MAb	Ascites	5200	335mg	68

Protein G affinity chromatography was applied to purify DC MAbs (in our case, all were IgGs). Protein G (30-35 KD) isolated from β-hemolytic Streptococci (group G) has a

natural affinity for the constant region of the antibody heavy chain (Fc). Anti-DC MAbs and anti-PSA MAb producing hybridomas originated from different species (rat, hamster, and mouse). Hybridomas produce IgG of different subclasses. Protein A does not have a comparable binding affinity or diversity in subclass binding that can be found in protein G [Bjorck L, 1990]. Our preliminary experimental results showed that protein G chromatography had higher efficiency than protein A chromatography. Thus, we chose protein G chromatography for purifying anti-DC MAbs. Table 2-2a and Table 2-2b are cited from the protein G Product Information Sheet from BIODESIGN Company as references.

Table 2-2a Protein A/G Affinities for Antibodies from Various Species

Species	Affinity for protein A	Affinity for protein G
Mouse	++	++
Rat	+/-	++
Hamster	+	++

Table 2-2b Protein A/G Affinities for Various Monoclonal Antibodies

Antibodies	Affinity for protein A	Affinity for protein G
Rat IgG2a	-	++++
Rat IgG2b	-	++++
Mouse IgG1	+	++++

Previous work in our laboratory demonstrated that large quantities of high purity anti-PSA MAbs could be obtained by these methods. The production and purification of anti-

PSA MAb were performed by ascites fluid formation in mice and protein G affinity chromatography.

Anti-DC MAbs and anti-PSA MAb were purified from cell culture supernatants and ascites fluids using protein G chromatography. After protein G chromatography, all purified MAb products from the same hybrid cell culture supernatants and ascites were pooled. The final concentration of the purified products was determined by ELISA (see Section 3.2.3.1.), as well as the measurement of UV absorbance at 280nm using a factor of $\varepsilon = 1.46$ for the antibodies.

The purity of the final MAb products was assessed by SDS-PAGE. Results showed that high purity of anti-DC MAbs and anti-PSA MAb was obtained by protein G chromatography. One light band appeared on the lane of DEC-205 MAb and 33D1 MAb at molecular weight about 66 KD. This might be the BSA from the standard media or other protein with similar molecular weight from the ascites (Figure 2-1a, 2-1b). Serum free media SFM and UD were employed for large-scale anti-DC hybrid cell culture. They were considered superior to standard medium in the follow-up antibody purification due to their low protein contamination. A SDS-PAGE assay showed minor contaminants in the final products. Standard medium can be a choice for a low-cost anti-DC MAb production from these hybrid clones (DEC-205, N418 and 33D1).

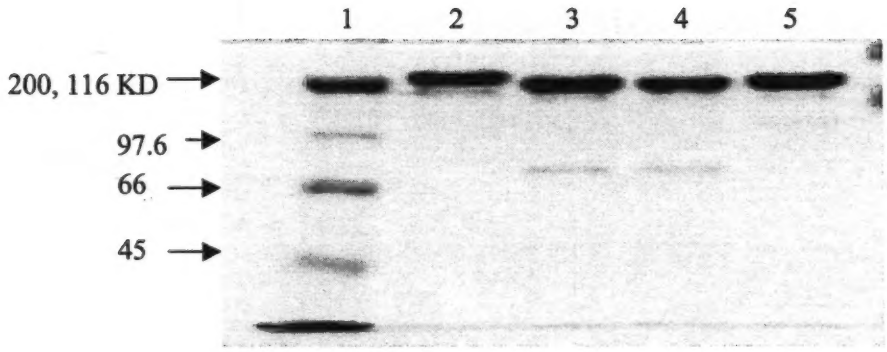


Figure 2-1a. SDS-PAGE Analysis of protein G chromatography purified MAbs under non-reducing conditions. Samples, 10μg each, were loaded in each well on a 7% SDS polyacrylamide Trizma-base gel. After electrophoretic migration the proteins were visualized by Coomassie blue. Lane 1: Broad range MW marker. Lane 2: N418 MAb. Lane 3: DEC-205 MAb. Lane 4: 33D1 MAb. Lane 5: Anti-PSA MAb.

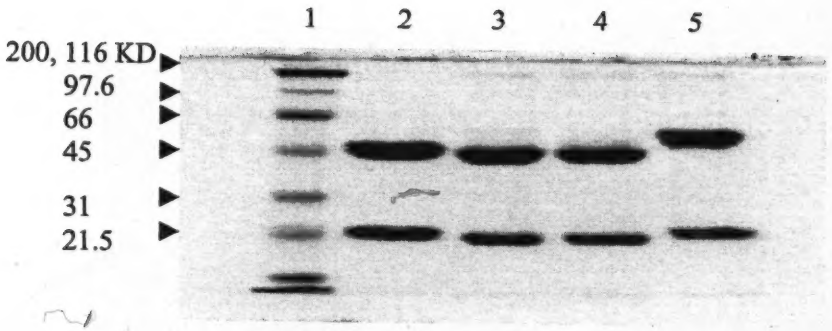


Figure 2-1b. SDS-PAGE Analysis of protein G chromatography purified MAbs under reducing conditions. A 10μg of samples were loaded on each well of a 10% SDS polyacrylamide Trizma-base gel. After electrophoretic migration the proteins were visualized by Coommassie blue staining. Lane 1: MW. Lane 2: Anti-PSA MAb. Lane 3: DEC-205 MAb. Lane 4: 33D1 MAb. Lane 5: N418 MAb.

2.2. Modification of proteins

The intention of the protein modifications was to optimize these proteins targeting to unique antigen-presenting dendritic cells. With optimized DC binding activity, these modified proteins would be taken up by DCs and degraded into peptides. The peptides would be presented on MHC molecules on the DC surface ready to react with antigen specific T cells. Induction of the antigen specific immune response was expected.

In our studies, anti-PSA MAb and PSA were the subjects of the modification. Chemical conjugation of anti-PSA MAb with anti-DC MAbs, mannosylation of anti-PSA MAb and PSA, mannan conjugation of anti-PSA MAb and PSA were attempted.

2.2.1. Chemical conjugation of bispecific agents

The bispecific agents referred to in this study are two MAbs with distinct binding specificities conjugated chemically. They belong to the family of bispecific antibodies (BsMAbs). BsMAbs do not occur naturally but can be created by different methods:

1) Chemically, cross-linking two MAbs with distinct specificities [Cook AG, 1994].
2) Biologically, somatic fusion of two hybridomas secreting the desired antibodies [Suresh MR, 1986].
3) Molecular engineering [Mack M, 1995; Coloma MJ, 1997].

Studies have indicated that both the specificities and affinities of the respective paratopes in BsMAbs are similar to those of the parental MAbs [Nolan O, 1990].

Chemical conjugations of anti-PSA MAb with three different anti-DC MAbs were conducted in our research. Generally, chemical linking can be achieved in two ways: 1) A direct coupling of the whole antibody molecules of two desired specificities or their Fab fragments [Glennie MJ, 1987, Cook AG, 1994]; 2) Dissociation and reassociation of heterologous immunoglobulins [Brennan, 1985]. Compared to biological methods, chemical production of BsMAbs has several advantages. First, the methods do not require cell fusion, which is quite time consuming. In addition, the desired BsMAbs are relatively easy to prepare in high yield [Cook AG, 1994], and they can be applied in most in vitro studies [Xu DZ, 1998].

In our research, glutaraldehyde was applied as a cross-linker for the conjugation of anti-PSA MAb and anti-DC MAbs. Glutaraldehyde can act as a homobifunctional reagent for coupling two proteins via their amine groups [Dent AH, 2001]. Glutaraldehyde reacts at ε-NH_2Lys, α-NH_2, and SH-Cys residues of MAb as primary sites. Also, it reacts randomly at Tyr and His residues as secondary reaction sites [Coligan JE, 1999]. This chemical conjugation method has the advantage of being rapid and simple. The presence of conjugated anti-PSA MAb with anti-DC MAb was analyzed by SDS-PAGE. SDS-PAGE results (Figure 2-2a) showed the conjugate bands were above a MW of 200- and 116 KD. An excess of glutaraldehyde led to a better production yields. Figure 2-2b showed the SDS-PAGE result of conjugated anti-PSA MAb with anti-DC (DEC-205 MAb) using two-fold of the cross linker. More efficient conjugation was observed.

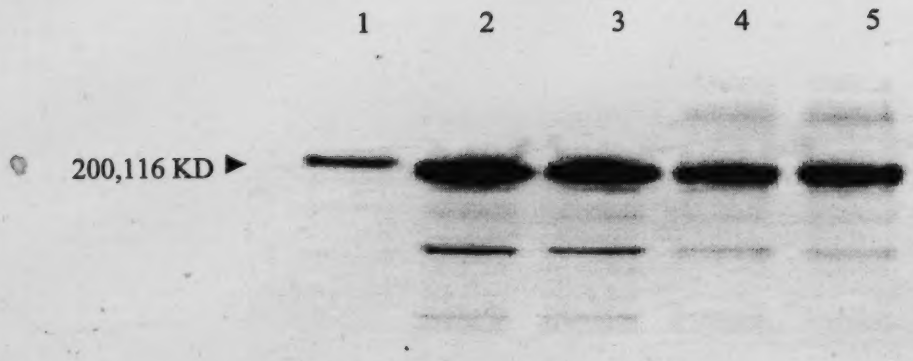


Figure 2-2a. SDS-PAGE Analysis Chemically conjugated anti-PSA MAb with anti-DC MAbs by using 0.3% glutaraldehyde. Samples, 10μg each without reducing agent, were electrophoresed on 4-15% SDS poly-acrylamide Tris-HCl Ready Gel. The proteins were visualized by staining with Coomassie blue. Lane 1: Anti-PSA MAb. Lane 2: Anti-DC MAb (DEC-205 MAb). Lane 3: Conjugated anti-PSA MAb with anti-DC MAb (33D1 MAb). Lane 4: Conjugated anti-PSA MAb with anti-DC MAb (DEC-205 MAb).

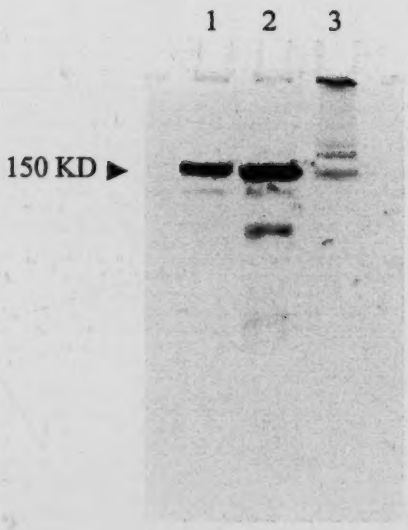


Figure 2-2b. SDS-PAGE Analysis Chemically conjugated anti-PSA MAb with anti-DC MAb (DEC-205) using 0.6% glutaraldehyde. Samples, 10μg each without reducing agent, were loaded on 4-15% SDS polyacrylamide Tris-HCl Ready Gel. The proteins were visualized by staining with Coomassie blue. Lane 1: Anti-PSA MAb. Lane 2: Anti-DC MAb (DEC-205). Lane 3: Chemically conjugated anti-PSA MAb with anti-DC MAb (DEC-205).

PSA binding activity of bispecific agents was assessed by ELISA, which is detailed in Section 3.2.3.2. The results (Figure 2-3) showed that bispecific agents had some PSA binding activity but this was achieved only at relatively high concentrations. The detection limit of bispecific agents was about 0.3μg/mL. The test concentrations of 33D1 conjugate were 0.3-58μg/mL, and for DEC-205 conjugate, were 0.3-65μg/mL. Preliminary investigation showed that bispecific agents which were conjugated with excessive cross-linker, had decreased PSA binding activity. PSA binding sites of anti-PSA MAb were definitely blocked during the conjugation by using a nonspecific cross-linker.

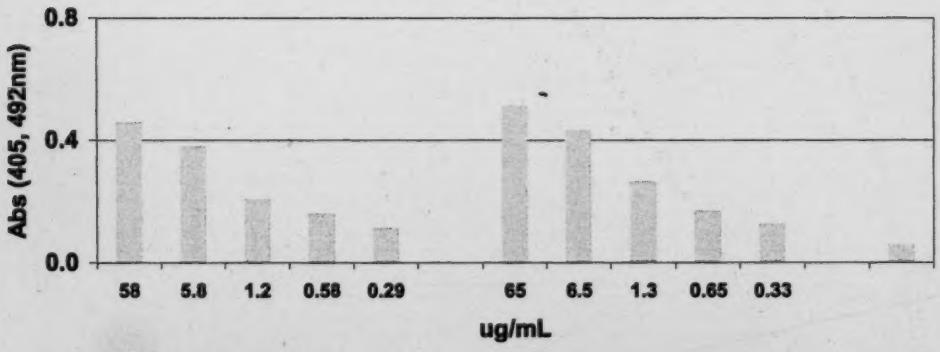


Figure 2-3. ELISA Result of PSA binding activity of bispecific agents (anti-PSA MAb conjugated with 33D1 MAb and DEC-205 MAb). The ELISA methodology is described in Section 3.2.3.2.

2.2.2. Induction of anti-PSA immune response by bispecific agents in vivo

Generation of an anti-PSA immune response induced by bispecific agents was investigated *in vivo*. DBA and Balb/c mice were immunized with bispecific agents followed by challenge with tumor cell lines P815-PSA or Line1-PSA.

Tumor cell line P815-PSA is a mouse mastocytoma cell line transfected with the PSA gene. Line1-PSA is a mouse lung carcinoma cell line transfected with the PSA gene. Both of the cells secrete PSA during the cell growth.

In our experiments, P815-PSA tumor cells were inoculated subcutaneously in DBA mice. Tumor formation was examined at the injection site after an appropriate interval. Line1-PSA cells were inoculated intravenously in Balb/c mice. The ultimate site of Line1-PSA cell growth was observed on the lungs. Ten test groups, with positive and negative controls, were assigned in DBA and Balb/c mouse models. The induction of the anti-PSA immune response by bispecific agents was evaluated by detecting anti-PSA Abs in mouse serum by ELISA. Tumor progress was assessed by taking the tumor weight in DBA mice and counting the tumor growth sites in the lungs of Balb/c mice. A detailed animal protocol is described in Section 3.2.3.8.

As therapeutic agents, the ideal conjugates of anti-PSA MAb and anti-DC MAb (with both PSA and DC binding activity) are expected to have the ability to induce a stronger anti-PSA immune response than that of the non-conjugated anti-PSA MAb alone. The anti-PSA immune response could be a cellular immune response specific to PSA, or a

humoral response which elicits production of anti-PSA Abs. Tumor growth progression would be evaluated by comparing the average tumor weight among DBA mouse groups, and by counting the lung colonies of each test group in Balb/c mice. But, in DBA mice, there was no significant difference in the average tumor weight of each test group. The tumor weights among individuals in each test group showed high variability and were therefore inconclusive; In Balb/c mice, the lung tumor colonies showed no significant difference among each test group. The evaluation of the induction of anti-PSA immune response by bispecific agents was therefore mainly based on the detection of anti-PSA Abs in the collected sera.

In this study, the detection of anti-PSA Abs in the sera was conducted by applying indirect ELISA. The following are the test groups involved in the animal experiments.

Negative control groups: 33D1 MAb alone (33D1)

DEC-205 MAb alone (DEC-205)

PBS

Positive control groups: Anti-PSA MAb alone

Anti-PSA MAb + PSA (anti-PSA MAb complex)

PSA alone

Tested groups: Conjugated anti-PSA MAb with 33D1 MAb (33D1 C)

Conjugated anti-PSA MAb with DEC-205 MAb (DEC-205 C)

Conjugated anti-PSA MAb with 33D1 MAb + PSA (33D1 complex)

Conjugated anti-PSA MAb with DEC-205 MAb + PSA (DEC-205 complex)

ELISA negative control: binding buffer or normal mouse serum.

ELISA positive control: anti-PSA MAb at concentration of 0.1μg/mL.

Figure 2-4a shows the ELISA result of the anti-PSA Abs in DBA mouse serum. Positive control groups showed a significant anti-PSA Ab response. Compared to the negative controls, 33D1 C showed a significant positive result, especially in the serum collected after tumor inoculation. DEC-205 C showed a positive result as well, but not as significant as 33D1 C. The 33D1 complex gave much stronger anti-PSA Ab response than 33D1 C alone, which was similar to the positive control groups. However the DEC-205 complex did not elicit a significant response compared to DEC-205 C alone. Anti-PSA Abs in each group, including negative control groups, showed a trend of increasing. This is most likely because human PSA secreted by inoculated tumor is immunogenic to mice and resulted in anti-PSA Ab production in the mouse's circulation.

Figure 2-4b summarizes the ELISA results for anti-PSA Abs detected in Balb/c mouse serum. As with DBA, positive control groups showed a significant anti-PSA Ab response. Compared to negative controls, 33D1 C and DEC-205 C showed a positive anti-PSA Ab response, but the response was not as strong as the positive control groups. Furthermore, the anti-PSA Abs in these groups tended to decrease. The 33D1 complex showed significant anti-PSA Ab response, compared to 33D1 C. In contrast, DEC-205 complex did not show an anti-PSA Ab response.

In general, anti-PSA Abs in the sera from both DBA and Balb/c models, 33D1 C had some capacity to induce an anti-PSA Ab immune response. Its Ag/Ab complex (33D1 complex) had higher capacity to induce anti-PSA Ab production. Compared to 33D1 C,

DEC-205 C had similar capacity to induce an anti-PSA Ab immune response. However, its Ag/Ab complex (DEC-205 complex) had little or no ability to induce anti-PSA Ab production.

In nearly all carefully studied instances, humoral and cellular immune immunity are mutually exclusive [Pilarski L, 1999]. Delayed type hypersensitivity (DTH) and cytotoxic T lymphocytes (CTL) refer to cellular mediated immunity (CMI). Humoral immunity is mediated by B cells which differentiate to secrete Abs of various isotypes. The immune class is regulated by the level of regulatory T cells in the system [Pilarski L, 1999]. Regulatory T cells influence all classes of immunity and circulate throughout the body. They secrete cytokines in the local environment within which inductive events occur. The T_H1 (subset of T helper cells) is responsible mainly for activation of CTLs and macrophages, and secretes IL-2, INF-γ, and TNF-β, etc. T_H2 is responsible for Ab secreting B-cell activation, and secretes IL-4, IL-5, IL-6 and IL-10, etc. [Kuby J, 1997]. The concentration of priming Ag and the immunogenic isotopes on the Ag are critical for the induction of certain types of immune responses [Pilarski L, 1999]. CTLs are believed to play critical roles in the control of destruction of tumor cells, and also in many viral infections [Hickling JK, 1998].

Our *in vivo* anti-PSA immune response study is summarized as follows:

(1) Detection of anti-PSA Abs alone did not provide sufficient evidence to conclude which type of anti-PSA immunity was induced in the animal experiments. The low concentration of anti-PSA Ab in the sera might be a possible sign of a CMI response.

This needs to be confirmed by further studies either analyzing cytokines from T-cell proliferation (IL-4, indication of humoral immune response or INF-γ, indication of CMI) or characterization of the population of T-cells ($CD4^+$ or $CD8^+$) in mice.

(2) Final products of bispecific agents applied in the animal experiments might contain a mixture of conjugated MAbs and free anti-PSA MAb, and anti-DC MAb as well. A suitable method has to be established for the purification of the bispecific agents. The cross linker was not specific. PSA binding activity of the conjugates was affected due to the cross linker used in the conjugation attached randomly to the functional groups of the proteins. This could cause the formation of anti-PSA MAb conjugated with anti-PSA MAb or anti-DC MAb conjugated with anti-DC MAb other than the ideal form (anti-PSA MAb conjugated anti-DC MAb). These might cause the actual concentration of bispecific agents lower than the ideal conjugates (anti-PSA MAb conjugated with anti-DC MAb) and might affect the result of the induction of anti-PSA immune response.

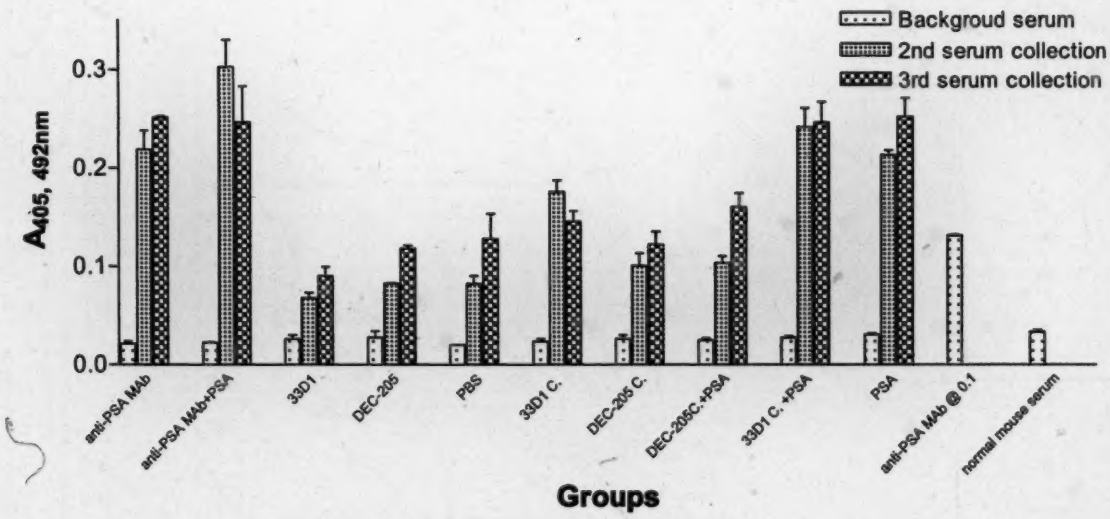


Figure 2-4a. Indirect ELISA Results of Anti-PSA Abs in DBA Mice

DBA mice were immunized with bispecific agents and 4.0 x 10^5 P815-PSA tumor cells were inoculated s.c. in each mouse. Sera were collected at appropriate time intervals. Indirect ELISA was applied to detect anti-PSA Abs in the sera. Tested sera were diluted 50 times. A detailed protocol of the animal work and ELISAs is described in Chapter 3 "Materials and Methods".

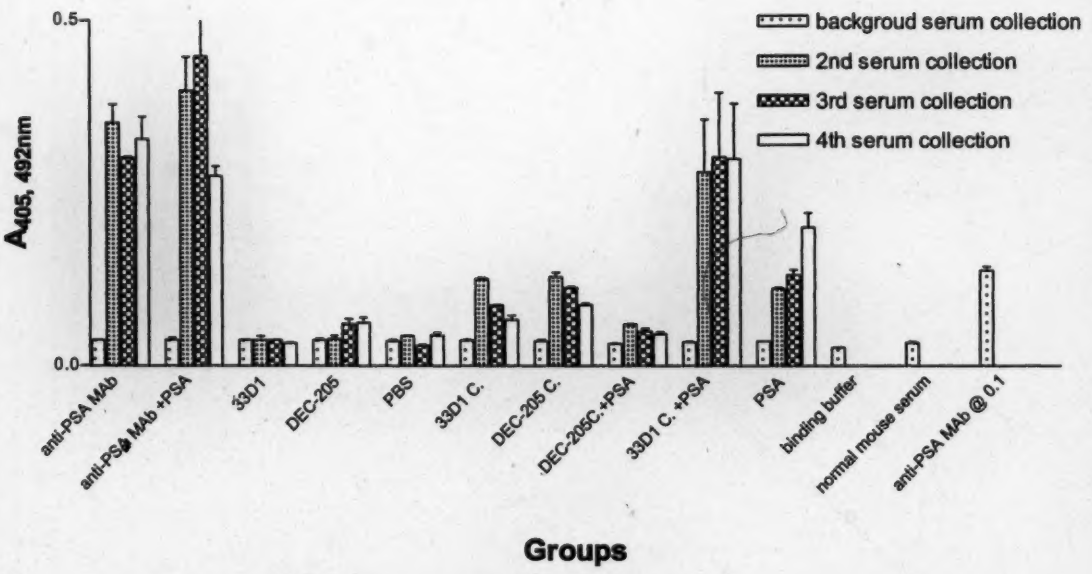


Figure 2-4b. Indirect ELISA Results of Anti-PSA Abs in Balb/c Mice

Balb/c mice were immunized with bispecific agents and 5.0 x 10^4 Line1-PSA tumor cells were inoculated i.v. Sera were collected at predetermined intervals. Indirect ELISA was applied to detect anti-PSA Abs in the sera. Test sera were diluted 50 times. Detailed schedule of the animal work and ELISA are described in Chapter 3 "Materials and Methods".

2.2.3. Mannosylation of anti-PSA MAb and PSA

DCs express high levels of mannose receptor that can mediate internalization of glycosylated ligands [Engering AJ (a), 1997]. There are numerous studies on the mechanism of mannose mediated antigen uptake as well as the uptake efficiency of mannosylated antigen compared with nonmannosylated antigen [Engering AJ (a), (b), 1997; Ogawara K, 1999; Agnes MC, 1997, 1998; Sallusto F, 1995; Apostolouposlos V, 1996, 2000]. Gene delivery through mannose mediated uptake has been explored. Plasmid DNA complexed with mannosylated liposomes exhibits high transfection activity due to recognition by mannose receptors both *in vitro* and *in vivo* [Kawakami S, 2000]. Mannosylpolyethyleneimine/DNA complexes containing adenovirus particles were successfully transfected into DCs. Such DCs are effective in activating T cells of T cell receptor transgenic mice in an antigen-specific fashion [Diebold SS, 1999].

In our experiments, mannosylated anti-PSA MAb and PSA were mannosylated *via* a phenylisothiocyanate bridge using α-D-mannopyranosylphenylisothiocyanate (α-D-M) in the presence of catalytic reagent N-methylmorpholine. To optimize mannosylating conditions, a 50x and a 500x of excess α-D-M were tested. A 200-fold excess of α-D-M was applied for mannosylation of PSA. Immobilized Concanavalin A (Con A) chromatography was applied for purification of mannosylated anti-PSA MAb. Results were compared by MBP chromatography. The chromatography conditions for Con A and MBP are listed in Table 2-3 and Table 2-4.

Table 2-3 Samples Loaded on the Con A Column

	Loading Amount	Total Volume Loaded	$A_{280\,nm}$
Anti-PSA MAb	540μg	2.5mL	0.370 (5x diluted)
Man-anti-PSA MAb (50x)	500μg	7.5mL	0.244 (no dilution)
Man-anti-PSA MAb (500x)	500μg	7.5mL	0.317 (no dilution)

Table 2-4 Samples Loaded on the MBP Column

	Loading Amount	Total Volume Loaded	$A_{280\,nm}$
Man-anti-PSA MAb (50x)	500μg	7.5mL	0.244 (no dilution)
Man-anti-PSA MAb (500x)	500μg	7.5mL	0.317 (no dilution)

Con A and MBP affinity chromatography are commonly used purification methods for glycoproteins. Immobilized Con A and MBP interact with proteins containing carbohydrate moieties of the correct configuration, with the mannan binding protein molecule [Nevens JR, 1992]. Immobilized Con A has been applied to purify glycoproteins from different sources, *e.g.* enzymes from brain [Brattain MG, 1977], membrane glycoproteins [Allan D, 1977], plasma proteins [Page M, 1973; Murthy RJ, 1973; Weinstein Y, 1972; Heimer R, 1978], hormones and hormone receptors [Dufau ML, 1972; Jacobs S, 1977; Printz MP, 1977]. MBP was recommended to purify IgM by the manufacturer (PIERCE). The purification efficacy of these two types of chromatography was evaluated by UV absorbance measurement and ELISA of the eluted fractions. The results (Figure 2-5) showed that the eluted fractions of mannosylated anti-PSA MAb contained active anti-PSA MAb from MBP chromatography (both at 50x excess of α-D-M and 500x excess of α-D-M), but not from Con A chromatography

(highly mannosylated product at 500x excess of α-D-M). Con A is more efficient for purifying mannosylated anti-PSA MAb than MBP chromatography in our case.

Linear gradient elution solution 0.1M – 0.5M of α-D-methylglucoside was applied to elute bound mannosylated anti-PSA MAb on immobilized Con A beads (Figure 2-6a, 2-6b, 2-6c). The less the degree mannosylated anti-PSA MAb, the weaker the binding activity on the Con A column. Results showed that different degrees of mannosylated anti-PSA MAb could be completely eluted at 0.1M - 0.3M of eluting solution (in the case of 500x excess of α-D-M). There were unbound anti-PSA MAbs in the wash solution of non-mannosylated anti-PSA MAb and mannosylated anti-PSA MAb (50x excess of α-D-M) but not in the wash solution of mannosylated anti-PSA MAb (500x excess of α-D-M). These results showed that the mannosylated anti-PSA MAb was obtained by adding at least a 50x excess of α-D-M for mannosylation. Fully mannosylated anti-PSA MAb could be achieved at 500x excess of α-D-M. Mannosylated PSA was purified by dialysis of the solution against PBS to remove free α-D-M and other chemicals applied in the reaction.

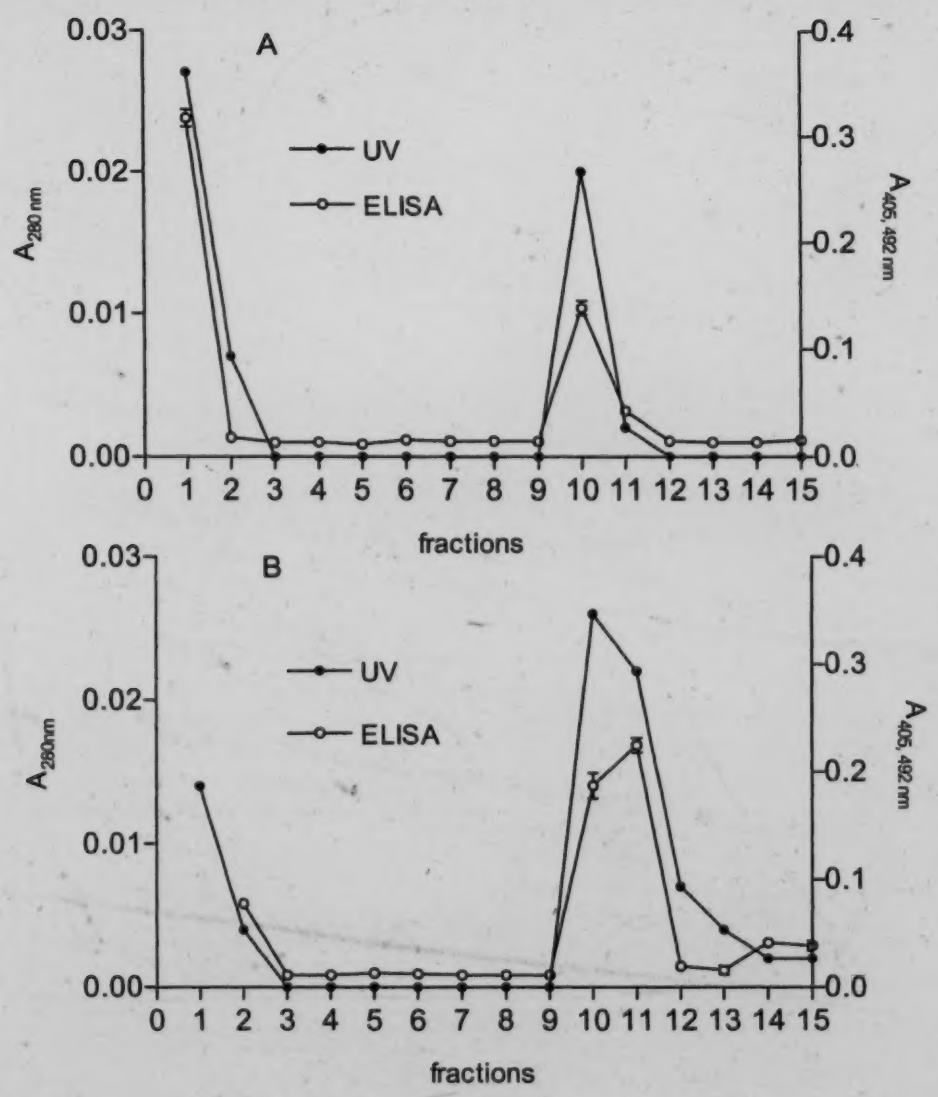


Figure 2-5. UV Absorbance and ELISA Results of eluted fractions of mannosylated anti-PSA MAb by MBP chromatography. Result of mannosylated anti-PSA MAb with excess 50x of α-D-M (A) compared with mannosylated anti-PSA MAb with 500x of α-D-M (B).

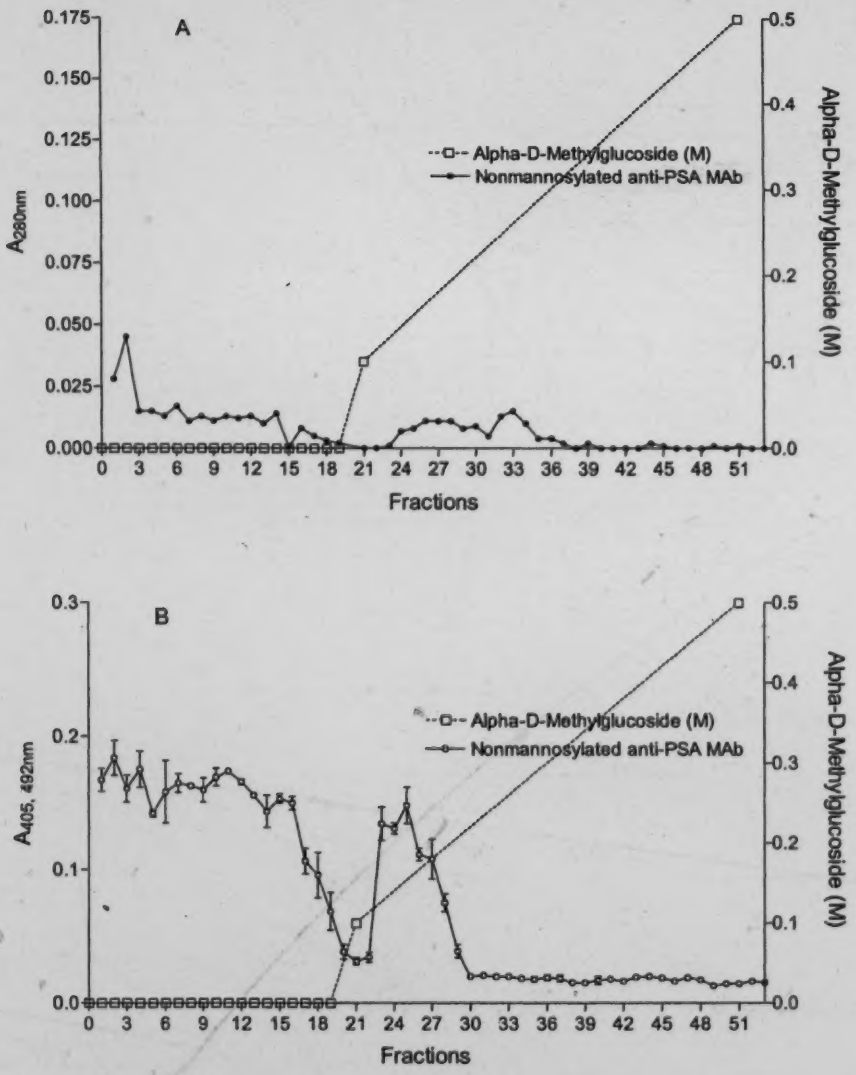


Figure-2-6a. UV Absorbance and ELISA Results Purified Non-mannosylated Anti-PSA MAb by Con A Chromatography

Non-mannosylated anti-PSA MAb was subjected to Con A chromatography in order to compare with mannosylated anti-PSA MAb on Con A chromatography. The eluted protein fractions were monitored by measuring the absorbance at 280nm (A) and by ELISA (B). Chromatography was performed by loading 500μg of anti-PSA MAb.

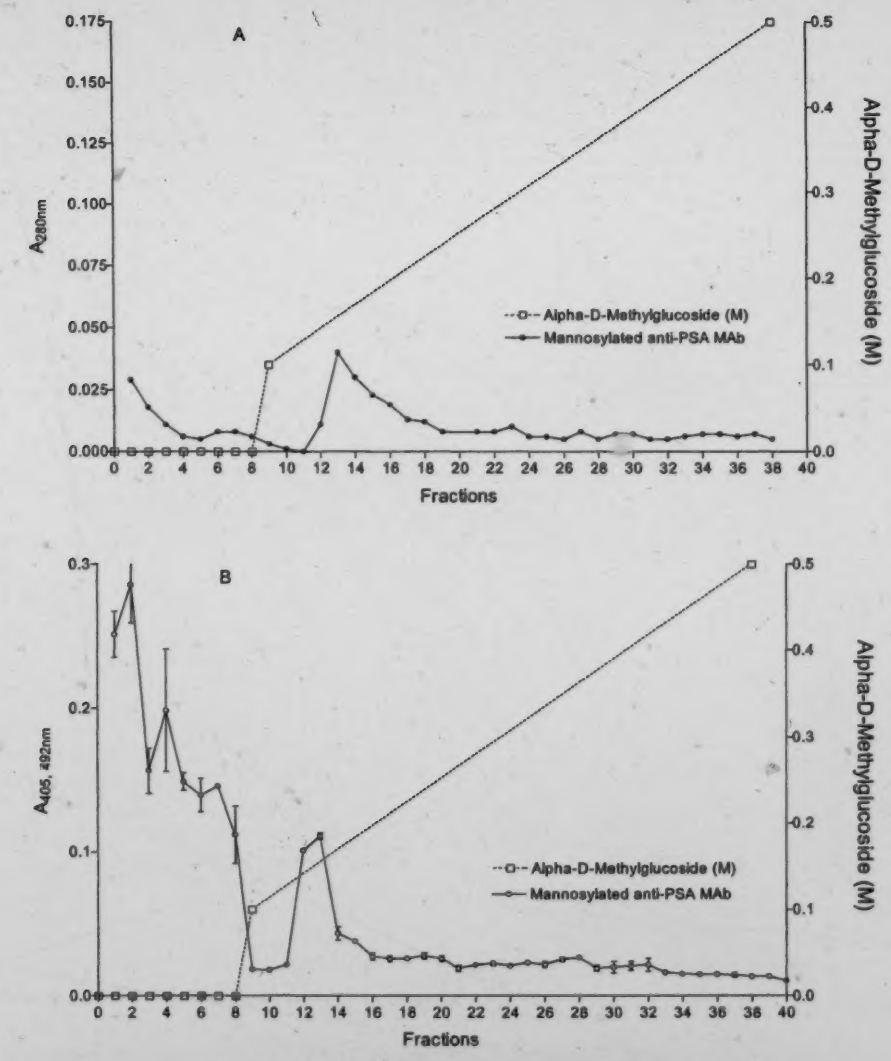


Figure 2-6b. UV Absorbance and ELISA Results Purified Mannosylated Anti-PSA MAb by Con A Chromatography

Anti-PSA MAb was mannosylated using a 50x excess of alpha-D-M as described in the section of Materials and Methods. Mannosylated anti-PSA MAb was subjected to Con A chromatography and the eluted protein fractions were monitored by measuring the absorbance at 280nm (A) and by ELISA (B). The chromatography was performed by loading 500µg of mannosylated anti-PSA MAb.

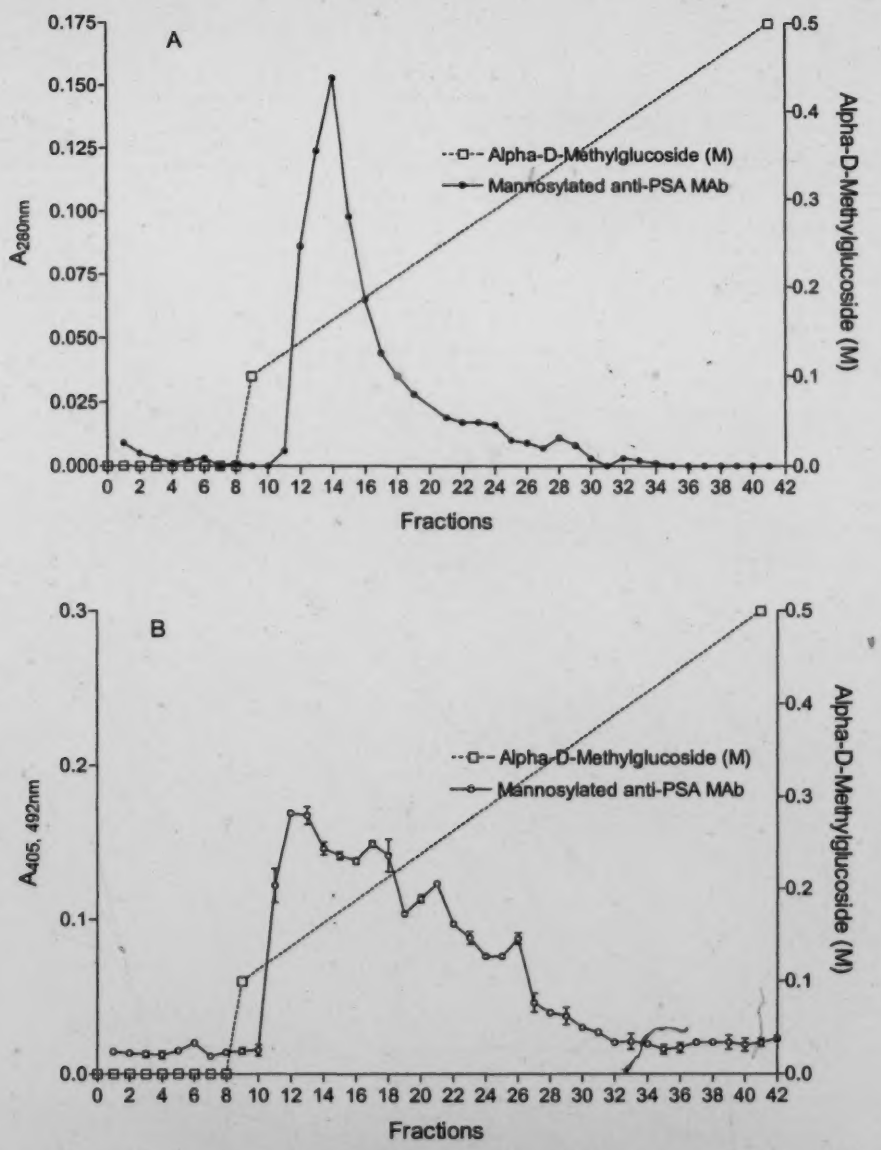


Figure-2-6c. UV Absorbance and ELISA Results Purified Mannosylated Anti-PSA MAb by Con A Chromatography

Anti-PSA MAb was mannosylated using a 500x excess of alpha-D-M as described in the section of Materials and Methods. Mannosylated anti-PSA MAb was subjected to Con A chromatography and the eluted protein fractions were monitored by measuring the absorbance at 280nm (A) and by ELISA (B). Chromatography was performed by loading 500μg of mannosylated anti-PSA MAb.

Since the concentration of mannosylated anti-PSA MAb eluted from Con A chromatography was very low (the estimated concentration was about 100μg/mL), silver staining was applied to detect mannosylated anti-PSA MAb and mannosylated PSA molecular weight changes. Non-mannosylated anti-PSA MAb and non-mannosylated PSA were used as detection references. Silver staining, which has a detection limit of 2-5ng/mL, possesses a better sensitivity than Coomassie blue staining [Coligan JE, 1999]. The silver staining results, shown in Figure 2-7, indicated that the molecular weight of mannosylated anti-PSA MAb on both heavy chain and light chain was slightly higher than that of nonmannosylated anti-PSA MAb. In the presence of reducing reagent (β-mercaptoethanol), no significant molecular weight change was observable for mannosylated anti-PSA MAb.

Silver staining demonstrated a few bands on the track of mannosylated PSA. This indicated that the PSA was partially mannosylated, which changed the molecular weight (of PSA) significantly. A 200x excess of α-D-M led to a close to complete PSA mannosylation.

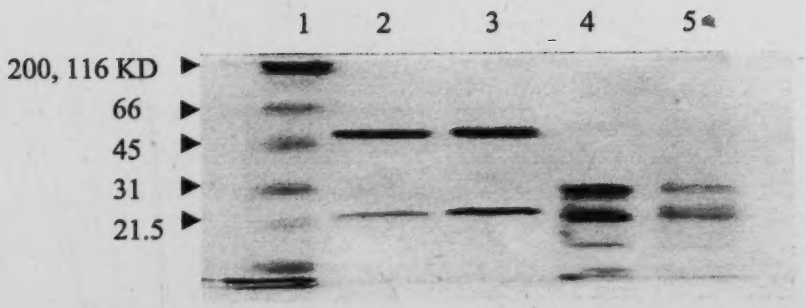


Figure 2-7. Silver Staining of mannosylated anti-PSA MAb (with reducing reagent) and mannosylated PSA (without reducing reagent). Samples were electrophoresed on 10% SDS poly-acrylamide Trizma-base gel. Lane 1: Broad range molecular weight marker. Lane 2: Non-mannosylated anti-PSA MAb. Lane 3: Mannosylated anti-PSA MAb. Lane 4: PSA. Lane 5: Mannosylated PSA.

Indirect ELISA (described in Section 3.2.3.2) was applied to evaluate the PSA binding activity of mannosylated anti-PSA MAb. According to the result of the indirect PSA binding assay shown in Figure 2-8, the PSA binding activity of mannosylated anti-PSA MAb was significantly decreased. The recognition of anti-mouse Ig(H+L) assay indicated that the binding activity of mannosylated anti-PSA MAb to anti-mouse Ig(H+L) HRP conjugate was significantly decreased as well. Thus, the PSA binding activity of mannosylated anti-PSA MAb was not detected by the indirect ELISA. Sandwich ELISA (detailed in Section 3.2.3.3.) may be a better choice to assess the true PSA binding activity of mannosylated anti-PSA MAb.

Fluorescence microscopy was applied to evaluate the DC binding activity of mannosylated proteins. A much higher fluorescence intensity on the murine dendritic cell surface was observed in the sample of mannosylated PSA. There was no significant difference between mannosylated anti-PSA MAb and non-mannosylated anti-PSA MAb.

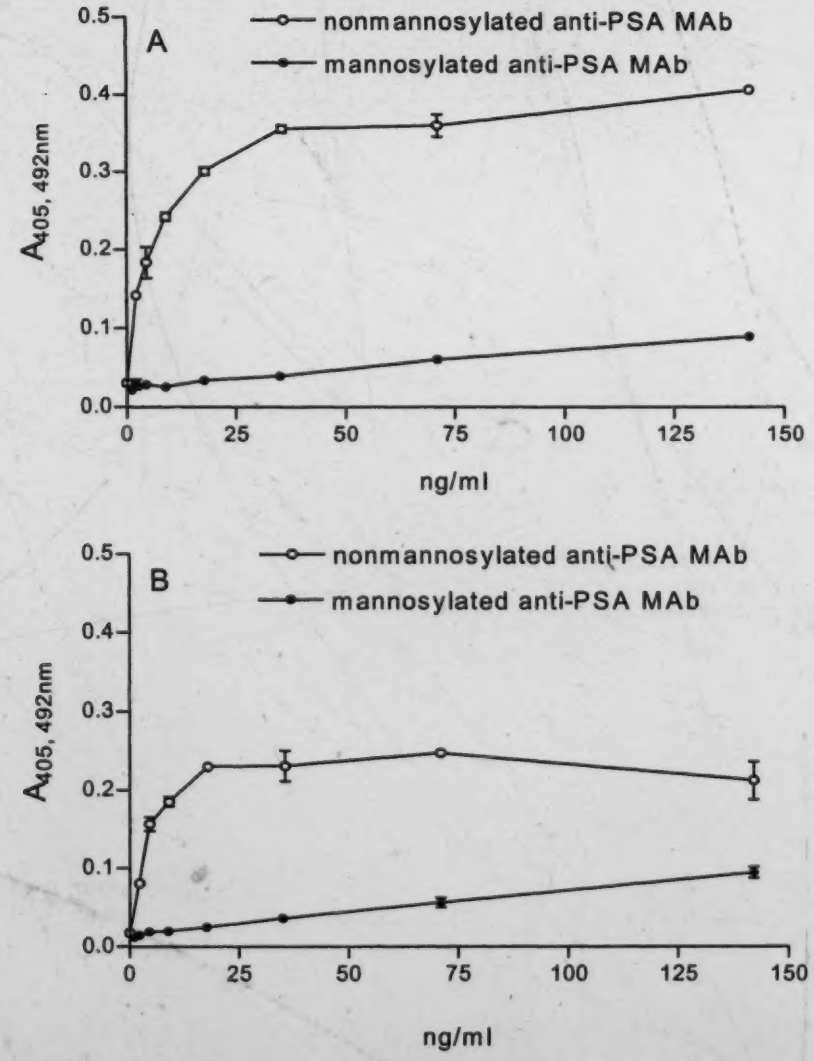


Figure 2-8. PSA Binding Assay of Mannosylated Anti-PSA MAb (500x α-D-M)

PSA binding activity of mannosylated anti-PSA MAb was evaluated by ELISA. Indirect PSA binding (A) and mouse Ig (H+L) binding (B) as described in Chapter 3, Materials and Methods.

2.2.4. Mannan conjugation of anti-PSA MAb

In vivo, a specific CTL immune response could be induced by oxidized mannan conjugated proteins [Apostolopoulos V, 1997]. *In vitro,* mechanistic studies revealed that oxidized mannan conjugated protein targeted the MHC class I antigen-presentation pathway. The antigen uptake was mannose receptor mediated [Apostolopoulos V, 2000].

We conducted mannan conjugation with anti-PSA MAb and PSA in order to find a potentially effective agent to induce an anti-PSA immune response against prostate cancer. For mannan conjugation, mannan was first oxidized by sodium periodate at pH 9.0 (as described in Section 3.2.2.3.). Aldehyde groups were generated at the positions where a vicinal diol (hydroxyl residues on two adjacent carbon atoms) was present [Dent AH, 2001]. The amino groups of the anti-PSA MAb react with the aldehyde residues of the oxidized mannan [Dent AH, 2001; Apostolopoulos V, 1996].

The mannan conjugated anti-PSA MAb was purified by dialysis against PBS at a molecular weight cutoff of 120,000 Da to remove free mannan (MW 34-62 KD) in the reaction solution. The dialyzed solution was analyzed by SDS-electrophoresis using Coomassie stain for protein visualization. SDS-PAGE (Figure 2-9) showed that anti-PSA MAb was fully mannan conjugated. There were faint bands at the Ab light chain position (MW 25KD). The PSA binding activity of mannan conjugated anti-PSA MAb was analyzed by ELISA. The indirect PSA binding assay showed a similar result to mannosylated anti-PSA MAb, with a significantly decreased PSA binding activity. The result of the sandwich ELISA for PSA binding assay was very encouraging. The mannan

conjugated anti-PSA MAb showed similar PSA binding activity to non mannan conjugated anti-PSA MAb (Figure 2-10a, 2-10b). The residual PSA binding activity of mannan conjugated anti-PSA MAb indicated that the method for mannan conjugation is applicable for modification of anti-PSA MAb.

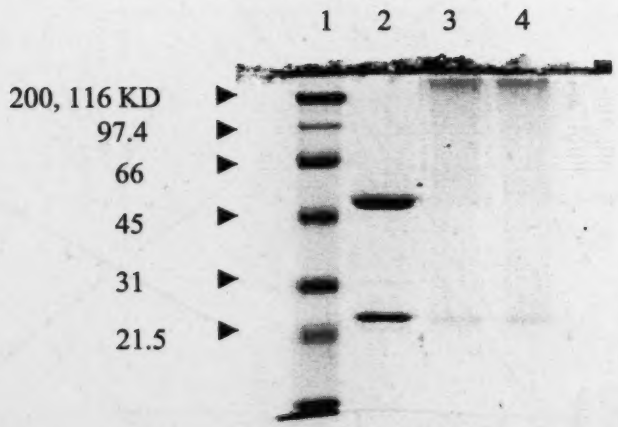


Figure 2-9. SDS-PAGE Analysis of Mannan-conjugated anti-PSA MAb under reducing conditions. Samples, 10μg each, were loaded in each well on a 10% SDS poly-acrylamide Trizma-base gel. After electrophoretic migration the proteins were visualized by Coomassie blue staining. Lane 1: Broad range molecular weight marker. Lane 2: Anti-PSA MAb. Lane 3: Oxidized Mannan conjugated anti-PSA MAb. Lane 4: Reduced mannan conjugated anti-PSA.

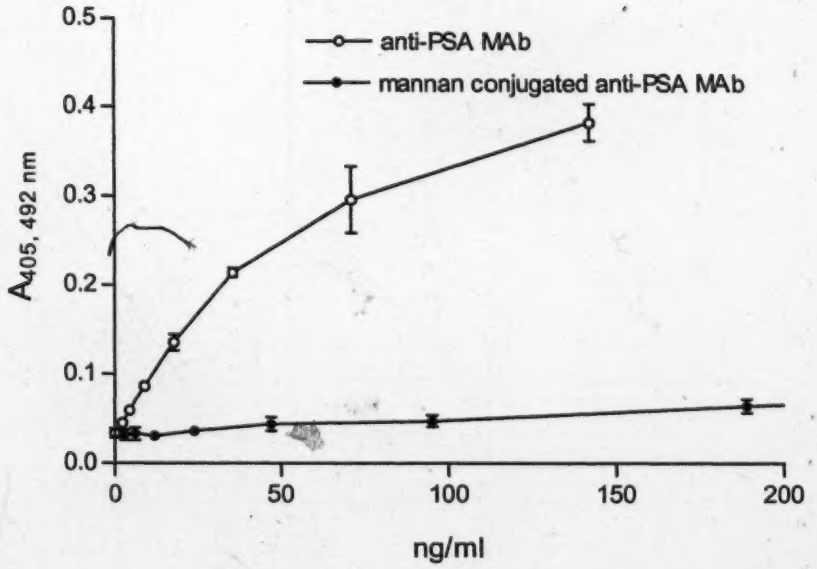


Figure 2-10a. Indirect ELISA Result for detecting PSA Binding Activity of mannan Conjugated anti-PSA MAb

PSA binding activity of mannan conjugated anti-PSA MAb was compared with free anti-PSA MAb in the indirect ELSA method. Detailed procedure is described in Section 3.2.3.1.

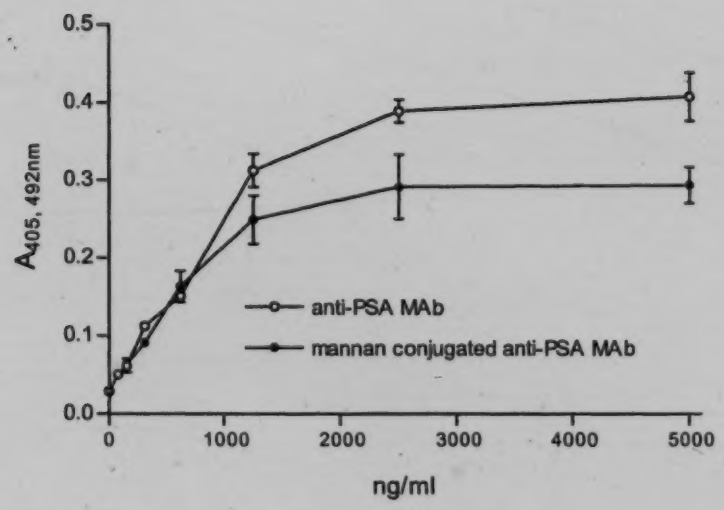


Figure 2-10b. Sandwich ELISA result for Detecting PSA Binding Activity of mannan Conjugated Anti-PSA MAb

PSA binding activity of mannan conjugated anti-PSA MAb was compared with free anti-PSA MAb in the Sandwich ELISA. Detailed procedure is described in Section 3.2.3.3.

2.3. Dendritic cell uptake assay

Radioisotope ^{125}I was applied as a tracer to evaluate the uptake of mannosylated PSA and mannan conjugated PSA by cultured murine dendritic cells.

The chloramine-T direct iodination method was used for iodination of PSA. Chloramine-T acted as an oxidizing agent, which converted iodide (I^-) to a more reactive form (I^+). $^{125}I^+$ targeted H^+ neighbouring the hydroxyl group on tyrosine aromatic ring and replaced the H^+. The reaction required the mixing of PSA with sodium iodide ($Na^{125}I$) followed by the addition of chloramine-T. The reaction was terminated by adding reducing reagent, sodium metabisulfite. This labeling procedure of iodination was relatively simple and rapid. A 10DG chromatographic column was used for the purification of iodinated PSA. This polyacrylamide gel allowed the separation of molecules according to their molecular weights. It excluded MW greater than 6,000Da. Free iodine was retained in the column. Thus, purification of ^{125}I-PSA was achieved.

The PSA mannosylation and mannan conjugation were performed following iodination of PSA to obtain comparable amounts of ^{125}I in each group. Mannosylated ^{125}I-PSA was purified by 10DG chromatography to eliminate the free mannose. No further purification was attempted for mannan conjugated ^{125}I-PSA. Mannose, 100μg/mL, was applied as mannose receptor inhibitor as a negative control.

The results (Figure 2-11) showed that background values (the negative control group of free ^{125}I) were negligible. Other groups showing positive values indicated that ^{125}I-PSA

and modified ^{125}I-PSA were taken up by dendritic cells. The result showed that all groups reached essentially the highest radioactivity at 15 minutes after the treatment. This result corresponded with an earlier publication [Engering AJ, 1997]. The mannosylated ^{125}I-PSA showed higher radioactivity than other groups, compared to the control group (mannosylated ^{125}I-PSA + mannose). Although the result was not statistically significant, it did indicate that mannosylation of protein increased the uptake by DC. Mannan conjugated PSA failed to show a positive result. One possible reason is that no purification for mannan conjugated PSA was applied after the modification. There was a possibility that a significant amount of free mannan existed in the product solution. Free mannan might compete with mannan conjugated PSA, and could bind to mannose receptors. Thus, the binding activity of mannan conjugated PSA could have been inhibited. Another possible reason could be that DC demonstrated constant antigen engulfment. The concentration of ^{125}I labeled protein was very low (10^{-5}ng/cell). The dominant uptake pathway in our experiment might not be mediated through mannose receptors only, but also through other pathways, such as phagocytosis.

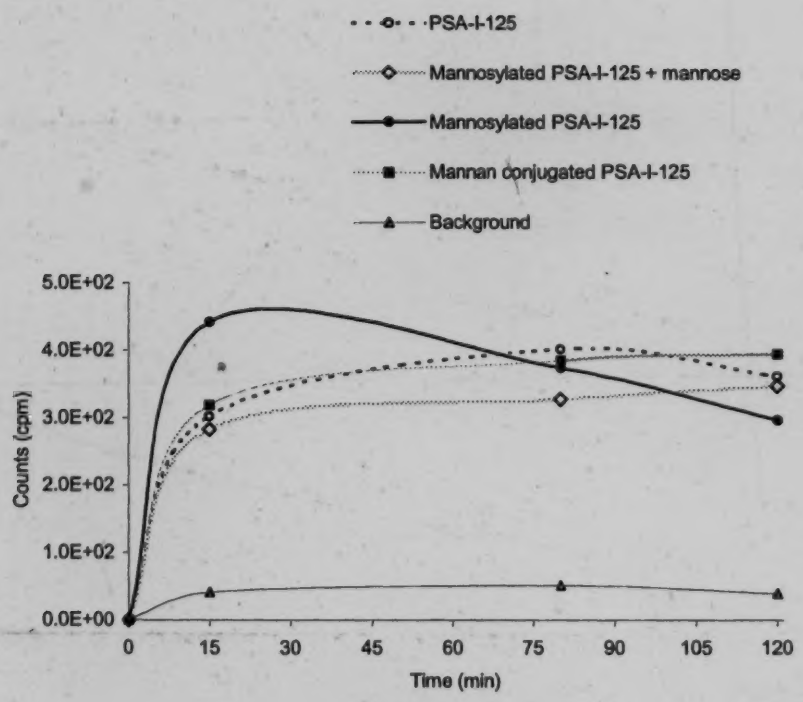


Figure 2-11. Result of Cultured Dendritic Cell Uptake Assay

PSA was iodinated before modification. Mannosylated ^{125}I-PSA and mannan conjugated ^{125}I-PSA of the same radioactivity were added in 2 x 10^5 DC. After appropriate incubation the radioactivity remaining in the cells was counted and compared with two control groups (non-modified ^{125}I-PSA and modified ^{125}I-PSA with inhibitor mannose).

CHAPTER 3

Materials and Methods

3.1. Materials

3.1.1. Cell lines and cell culture materials

Tumor cell lines (Line 1-PSA, P815-PSA), DC line (syn. JAWSII) and hybridomas (DEC-205, 33D1 and N418) were purchased from The American Type Culture Collection (ATCC, Manassas, VA, USA). RPMI-1640 medium, FBS, L-glutamine, streptomycin, penicillin, α-Minimum Essential Medium (α-MEM) with ribonucleosides and deoxyribonucleosides, Hank's Balanced Salt Solution (HBSS), and hybridoma serum-free medium (SFM) were obtained from Invitrogen Canada, Inc. (Burlington, ON, Canada). Ultradoma medium (UD) was obtained from BioWhittaker Inc. (Walkersville, MD, USA). Murine GM-CSF was obtained from PeproTech Inc. (Rocky Hill, NJ, USA).

3.1.2. Animals

SCID Beige mice, DBA mice and Balb/c mice were obtained from Health Sciences Laboratory Animal Services (University of Alberta, Edmonton, AB, Canada). All procedures were conducted according to the Guidelines of the Canadian Council on Animal Care.

3.1.3. Chromatography Columns

GammaBind Plus Sepharose (immobilized recombinant protein G sepharose) was purchased from Amersham Pharmacia Biotech, Inc. (Piscataway, NJ, USA).

Concanavalin A immobilized on beaded agarose was obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). The Mannan Binding Protein (MBP) column was obtained from PIERCE (Rockford, IL, USA). A 10DG desalting column was obtained from BioRad Laboratories (Mississauga, ON, Canada).

3.1.4. Antibodies

Goat anti-mouse Ig(H+L)-UNLB, Goat anti-mouse Ig(H+L)-HRP, Goat anti-rat Ig(H+L)-HRP, Anti-rat IgG, Anti-hamster IgG, and Hamster IgG were obtained from Southern Biotechnology Associates Inc. (Birmingham, AL, USA). Anti-hamster IgG-HRP and Goat anti-hamster IgG(H+L) FITC conjugate were obtained from Kirkegaard and Perry Laboratories (KPL), Inc. (Gaithersburg, MD, USA). Rabbit anti-rat IgG(H+L) FITC conjugate, Goat anti-mouse polyvalent Igs (IgA, IgG, IgM) FITC conjugate and Goat anti-rabbit IgG(H+L) FITC conjugate and Rat IgG were bought from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Rabbit anti-PSA IgG was obtained from BIODESIGN International (Saco, ME, USA).

3.1.5. Chemicals, reagents and accessories

Pristane (2,6,10,14-tetramethyldecanoic acid), α-D-mannopyranosylphenylisothiocyanate (α-D-M), and N-methylmorpholine (NMM), glycerol and mannan were obtained from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). PSA was obtained from SCRIPPS (San Diego, CA, USA). Ultrafiltration membranes YM-30 (MW cutoff 30,000 Dalton) and the Amicon concentration cell model-8200 were obtained from Amicon Bioseparations Inc. (Beverly, MA, USA). VacuCap filters (0.22μm) were obtained from

Gelman Sciences (Ann Arbor, MI, USA). NUNC microtitre strips were obtained from Invitrogen Canada, Inc. (Burlington, ON, Canada); 4-15% polyacrylamide Tris-HCl ready gels, 40% polyacrylamide, and prestained broad range molecular weight markers were obtained from BioRad Laboratories (Mississauga, ON, Canada). Sodium periodate was purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada). Sodium borohydride was purchased from Fisher Scientific Ltd. (Nepean, ON, Canada). ABTS (H_2O_2 solution and ABTS peroxidase substrate) were obtained from Invitrogen Canada, Inc. (Burlington, ON, Canada). Eight-chamber glass slides were FALCON brand purchased from VWR International (Edmonton, AB, Canada). ^{125}I was obtained from New Life Science Products Inc. (Boston, MA, USA). Analytical grade chemicals such as sodium iodide, chloramine-T, sodium metabisulfite, sodium periodate and all other chemicals were purchased from Sigma-Aldrich Canada Ltd. (Oakville, ON, Canada) and Fisher Scientific Ltd. (Nepean, ON, Canada).

3.2. Methods

3.2.1. Production of MAbs

3.2.1.1. Ascites production

Six-week-old male mice (Balb/c or SCID beige mice) were injected intraperitoneally with 0.7mL of Pristane. One week later 5 x 10^6 hybridoma cells suspended in 2mL of PBS were administered intraperitoneally. Ascites fluid was tapped from the mice in due course. Ascites was centrifuged at 1500x g for 10 minutes. The supernatant was stored at 4°C until all collections were completed. The collected fluids were stored at –20°C for subsequent MAb purification.

3.2.1.2. Hybridoma cell culture

Hybridoma cell lines 33D1, N418, and DEC-205 were cultured in standard medium (RPMI-1640 medium supplemented with 10% v/v FBS, 2mM L-glutamine, 50U/mL penicillin and 50U/mL streptomycin), hybridoma-SFM, and UD, respectively. The culture was maintained in a humidified incubator with 5% CO_2 at 37°C. Supernatants were collected and stored at -20°C for later use.

3.2.1.3. Purification of MAbs

Concentration of hybridoma cell culture supernatants

Hybridoma cell culture supernatants were first filtered through a 0.22μm filter and then concentrated by using an Amicon ultrafiltration cell. Before loading the supernatant, the membrane was placed in the Amicon ultrafiltration cell and rinsed with distilled water for 5 minutes at 20psi (1.37Atm). The supernatants were then loaded into the cell, and the flow rate was controlled under a maximum pressure of 40psi (2.74Atm), until the desired volume was achieved.

Protein G affinity chromatography

The column, containing 5mL of immobilized protein G sepharose, was washed with 2 column volumes of elution buffer (0.5M acetic acid, pH 3.0). The column was then equilibrated with 5 column volumes of binding buffer (0.01M Na_2PO_4, 0.15M NaCl, and 0.01M EDTA; pH 7.0). The concentrated cell culture supernatants were diluted 1:1 v/v with binding buffer. Ascites fluid samples were diluted 1:10 v/v with binding buffer.

Diluted supernatants or ascites was loaded to the column at a flow rate of 0.5mL/min, and the column was washed with 50mL of binding buffer at a flow rate of 1.5mL/min. The absorbance of the collected fractions containing unbound proteins was monitored by spectrophotometer at 280nm. The proteins bound on the column were then eluted with 15mL of elution buffer. Eluted fractions were collected at 1.5mL per tube at a flow rate of 1mL/min. The pH of eluted fractions was immediately neutralized to 7.0 with 1M Trizma-base (pH 9.0). Eluted fractions of bound proteins specific for mouse, rat or hamster were subjected to ELISA. Detailed ELISA methods are outlined in Section 3.2.3.1.

Titration of purified MAbs by ELISA

Detailed method is described in Section 3.2.3.1.

Purity assay by SDS-PAGE

Detailed method is described in Section 3.2.3.5.

3.2.2. Modification of MAbs and PSA

3.2.2.1. Chemical conjugation of anti-PSA MAb with anti-DC MAb

Purified anti-PSA MAb and anti-DC MAb were mixed in equimolar concentration. The mixture was dialyzed against borax buffer (freshly prepared by mixing 57.5mL of 0.05M sodium borate solution with 25mL of 0.2M boric acid solution, volume-adjusted to 1000mL with DD water; pH 9.2) at 4°C. One-hundred μL of 0.3% glutaraldehyde solution was then slowly added to the dialyzed solution and the mixture was stirred at

room temperature for 2 hours. The reaction was stopped by adding 250µl of 1M glycine. After 30 minutes incubation under stirring, the mixture was dialyzed against PBS at 4°C.

3.2.2.2. Mannosylation of anti-PSA MAb and PSA

The protein, α-D-mannopyranosylphenylisothiocyanate (α-D-M), and N-methylmorpholine were mixed in the proportions described in Table 3-1. The mixture was stirred overnight at room temperature, and the excess α-D-M was hydrolyzed by addition of 100µL of 1M Trizma-base (pH 9.5). The solution was stirred for 1 hour at room temperature and then dialyzed against PBS to remove non-conjugated mannose residues at 4°C.

Table 3-1 Protein Mannosylation - Experimental Conditions

Mannosylation of anti-PSA MAb in the presence of 500x excess α-D-M					
	Concentration (M)	Mass (µg)	Volume (µL)	Reaction Volume (mL)	Volume after dialysis (mL)
Anti-PSA MAb	6.7×10^{-9}	1000	700	10.0	15
α-D-M	3.4×10^{-6}	1000	10		
NMM			2		
PBS			9300		
Mannosylation of anti-PSA MAb in the presence of 50x excess α-D-M					
Anti-PSA MAb	6.7×10^{-9}	1000	700	10.0	15
α-D-M	3.4×10^{-7}	100	1		
NMM			2		
PBS			9300		
Mannosylation of PSA in the presence of 200x excess α-D-M					
PSA	3.0×10^{-8}	100	37	0.5	3
α-D-M	6.0×10^{-6}	100	1		
NMM			2		
PBS			460		

Concanavalin A affinity chromatography

Immobilized Concanavalin A beads, 1mL in volume, were poured into a glass column. The column was prewashed with 20mL binding buffer (20mM Trizma-base, 0.5M NaCl, 1mM $CaCl_2$, 1mM $MnCl_2$; pH 7.4). Mannosylated anti-PSA MAb was loaded on the column at room temperature. Unbound proteins were washed out with 30mL of binding buffer. Bound proteins were eluted with a 30mL (0.1M - 0.5M) of α-D-methylglucoside, with a linear gradient. The eluted fractions were collected, in 1mL aliquots. The eluted fractions were monitored by measuring the absorbance at 280nm for the presence of proteins, and also by ELISA for the presence of murine immunoglobulin. Detailed ELISA procedures are described in Section 3.2.3.1.

MBP affinity chromatography

A Mannan Binding Protein (MBP) column, 5mL, was prewashed with 2 column volumes of elution buffer (10mM Trizma-base, 1.25M NaCl, 2mM EDTA; pH 7.4), and re-equilibrated with 20mL binding buffer (10mM Trizma-base, 1.25M NaCl, 20mM $CaCl_2$; pH 7.4). The sample was loaded on the column and incubated for 30 minutes at 4°C. The column was washed with 9 column volumes of the binding buffer to remove unbound proteins. Elution buffer, 1.5mL, or enough to soak the gel in the column, was added to the column. The column was incubated at room temperature for 1 hour and subsequently washed with elution buffer. Eluted fractions were collected in 3mL aliquots. The eluted fractions were monitored by measuring the absorbance at 280 nm for the presence of proteins, and by ELISA for the presence of mannosylated anti-PSA MAb. Detailed ELISA methods are described in Section 3.2.3.1.

3.2.2.3. Mannan conjugation of anti-PSA MAb and PSA

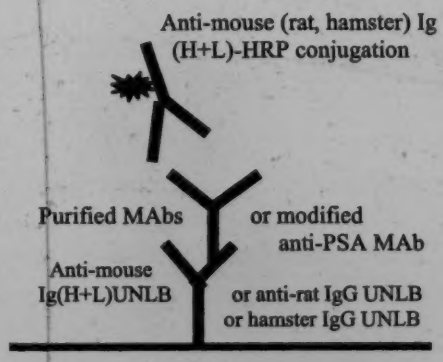
Mannan, 14mg, was dissolved in 1mL of 0.1M phosphate buffer (pH 6.0). Freshly prepared 0.1M sodium periodate, 100μL, was added and the mixture was stirred at 4°C for 60 min. Glycerol, 10μL, was added and incubated for 30 min at 4°C. The mixture was passed through a 10DG desalting column, which equilibrated in 0.1M bicarbonate buffer (pH 9.0). The oxidized mannan was collected in the void volume and mixed with 900μg of anti-PSA MAb or 200μg of PSA. The mixture was incubated overnight at room temperature with gentle stirring. No further purification was performed.

3.2.3. Characterization of modified anti-PSA MAb and PSA

3.2.3.1. Indirect ELISA I

This ELISA was applied for titration of purified MAbs, monitoring the presence of purified MAbs, mannosylated anti-PSA MAb in the eluted fraction from protein G, Con A, and MBP chromatography.

Microtitre strips were coated with 100μL of 5μg/mL of capture antibody [anti-mouse Ig(H+L) UNLB, or anti-rat IgG, or anti-hamster IgG] and incubated overnight at 4°C. The coating solution was discarded and the strips were blocked with buffer (2% sucrose, 3% BSA in PBS) to block non-specific binding sites, 150μL per well, for 1 hour. The strips were then washed three times with PBST (0.01% Tween in PBS) and incubated with 100μL of purified MAbs, or mannosylated anti-PSA MAb, or antibody standards (mouse or rat or hamster IgG). After

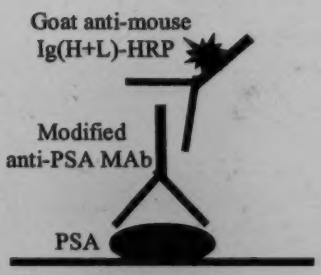


1-hour incubation, the strips were washed three times with PBST. The strips were then incubated with 100μL of HRP conjugated antibodies (anti-mouse or anti-rat or anti-hamster) at the dilution recommended by the manufacturers, followed by three washes with PBST. The activity of bound MAbs was determined by adding 100μL of ABTS (1:1 v/v dilution of H_2O_2 solution and ABTS substrate solution) for color development. The absorbance of each well was measured at dual wavelengths of 405nm and 492nm.

With the exception of the coating step, all remaining procedures were performed at room temperature.

3.2.3.2. Indirect ELISA II (PSA binding assay)

PSA solution, 0.5μg/mL in PBS, was immobilized on the microtitre strips, 100μL per well. The strips were incubated overnight at 4°C. The unbound sites were then blocked with blocking buffer (3% BSA, 2% sucrose in PBS), 150μL each well, for 1 hour.



Mannosylated or mannan-conjugated anti-PSA MAb was diluted 10-fold with binding buffer (1% BSA in PBS). Then 100μL of diluted sample was added into each well. Each sample was analyzed in duplicate. The strips were incubated for 1 hour and then washed three times with PBST (0.01% Tween in PBS). One-hundred μL of Goat anti-mouse Ig(H+L)-HRP which was diluted according to the manufacturer's instructions was then added to each well and the strips were incubated for 1 hour followed by washing with PBST three times. ABTS (freshly prepared by mixing equal amount of ABTS substrate

solution and H_2O_2) was added for color development, 100μL per well. The absorbance was measured at dual wavelengths of 405nm and 492nm.

With the exception of the coating step, all the remaining procedures were performed at room temperature.

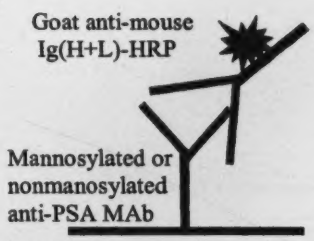
3.2.3.3. Sandwich ELISA for PSA binding

Microtiter strips were coated with 10-fold serial diluted anti-PSA MAb and mannan-conjugated anti-PSA MAb, 100μL per well. The strips were incubated at 4°C overnight. The unbound sites were blocked with 150μL blocking buffer (3% BSA, 2% sucrose in PBS), and the plate was incubated for 1 hour. Then 100μL of 0.5μg/mL PSA was added to each well, incubated for 1 hour, followed by washing three times with PBST. An 100μL aliquot of 1:10,000 diluted rabbit anti-PSA IgG was added to each well, and the strips were incubated for 1 hour. After three washes with PBST, Goat anti-rabbit Ig(H+L)-HRP conjugate, diluted according to the manufacturer's instructions, was added into each well. The strips were incubated for 1 hour and then washed three times with PBST. ABTS (freshly prepared by mixing equal amount of ABTS substrate solution and H_2O_2) was added for the color development, 100μL per well. The absorbance was measured at dual wavelengths of 405nm and 492nm.

With the exception of the coating step, the remaining procedures were performed at room temperature.

3.2.3.4. Recognition of mannosylated anti-PSA MAb by anti-mouse Ig(H+L)-HRP conjugates

Microtiter strips were coated with 10-fold serial dilutions of mannosylated or non-mannosylated anti-PSA MAb, 100μL per well, overnight at 4°C. The unbound sites were then blocked with buffer (3% BSA, 2% sucrose in PBS), 150μL per well. The strips were incubated for 1 hour and then washed three times with PBST. Goat anti-mouse Ig(H+L)-HRP diluted according to the manufacturer's instructions, was then added to each well, 100μL per well. The strips were incubated for 1 hour followed by three washes with PBST. ABTS (freshly prepared by mixing equal amounts of ABTS substrate solution and H_2O_2) was added for color development, 100μL per well. The absorbance was measured at dual wavelengths of 405nm and 492nm.



With the exception of the coating step, all the remaining procedures were performed at room temperature.

3.2.3.5. SDS-PAGE

SDS-PAGE was used to check the purity of the purified MAbs, and molecular changes of modified anti-PSA MAb.

Gel preparation was based on the detailed protocol in the Lab Manual of "Basic Immunology, 2000" [Faculty of Biological Sciences, Microbiology, University of Alberta]. Samples were diluted 1:1 v/v in sample buffer [0.5M Tris-HCl at pH 6.8, 20%

glycerol, 10% w/v SDS, 0.5% w/v bromphenol blue and 10% v/v β-mercaptoethanol (optional)]. The samples were heated for 4 minutes at 95°C in a waterbath. The samples, 25μL/well, were then loaded on a polyacrylamide gel. The gel was run in 1x running buffer (0.3% Trizma-base, 1.45% glycine, 0.1% SDS, pH 8.3) at 200V (constant voltage) for about 1 hour. The gel was then stained in Coomassie blue stain solution (0.1% w/v Coomassie brilliant blue, 40% v/v methanol, 10% v/v acetic acid in DD water) for about 2 hours with slow agitation. The gel was destained in 40% v/v methanol, 10% v/v acetic acid in DD water until the background color was faint and the blue bands of proteins were clearly visible.

3.2.3.6. Silver staining for the detection of mannosylated anti-PSA MAb and PSA

SDS-PAGE gels were fixed by successive incubations: first, in 50% v/v methanol, 10% v/v acetic acid in DD water for 40 minutes; second, in 5% v/v methanol, 7% v/v acetic acid in DD water for 60 minutes; and finally, in 10% glutaraldehyde for 30 min in a fume hood. The gel was then washed with DD water 4 times, 40 minutes each. Silver nitrate solution was freshly prepared by adding 3.5mL 30% NH_4OH to 42mL of 0.36% w/v NaOH. The final volume of the solution was brought to 200mL with DD water. Silver nitrate, 8mL of 19.4% w/v, was then added dropwise with stirring. The gel was stained with the silver nitrate solution for 15 minutes under vigorous shaking. The gel was then washed with deionized water 5 times, 1 minute each. The gel was developed in a developing solution (0.5% w/v sodium citrate, 0.5% v/v of 37% formaldehyde in DD water) with vigorous shaking until brown bands of proteins appeared. The gel was fixed

in destaining solution (5% v/v methanol, 7% v/v acetic acid in DD water) with slow agitation at room temperature for 10 minutes and at 4°C overnight.

3.2.3.7. Fluorescence microscopy for detection of DC binding activity of mannosylated anti-PSA MAb and PSA

Murine dendritic cell line culture procedures were followed according to the ATCC Product Information Sheet for CRL-11904 (JAWSII Mouse Dendritic Cell). Dendritic cells were grown in Complete Growth Medium, which is α-Minimum Essential Medium (α-MEM) with ribonucleosides and deoxyribonucleosides, containing 4mM L-glutamine, 1mM sodium pyruvate, and 5ng/mL murine GM-CSF. The medium was supplemented with 20% FBS. The cells were cultured in a humidified incubator at 37°C with 5% CO_2. The culture medium was renewed once a week.

Murine dendritic cells were seeded on an 8-chamber glass slide at a concentration of 1.25 x 10^5 cells/mL, 0.5mL per chamber, and cultured for two days. The culture medium was removed and the cells were incubated with 100μL of non-mannosylated anti-PSA MAb (5μg/mL), mannosylated anti-PSA MAb (5μg/mL), PSA (1μg/mL), or mannosylated PSA (1μg/mL), at 4°C for 45 minutes. Test antibodies were diluted in 5% mouse serum. Rabbit anti-PSA IgG, diluted 10,000 times, was added only into the chambers containing PSA to be tested, 100μL per chamber. The cells were washed twice with 300μL of 4°C HBSS and then incubated with 100μL of detective antibodies [anti-mouse polyvalent Igs FITC conjugate or anti-rabbit IgG(H+L) FITC conjugate], and diluted in HBSS according to the manufacturer's instructions. The cells were then washed three times with

300μL HBSS. The slides were coverslipped with glycerol/PBS (1:1 v/v). Fluorescent cells were photographed in the fluorescence microscope using a 100x oil immersion objective.

3.2.3.8. Induction of anti-PSA immune response by bispecific agents in vivo

Tumor cell culture

Tumor cell lines, Line1-PSA and P815-PSA, were routinely cultured in standard medium (RPMI-1640 medium supplemented with 10% v/v FBS, 2mM L-glutamine, 50U/mL penicillin and 50U/mL streptomycin). The cultures were maintained in a humidified incubator with 5% CO_2, at 37°C, until the cultures reached the desired cell density.

Immunization

Male Balb/c or DBA mice (age 5-6 weeks) were divided into 10 groups of 5 mice each. Each group was subjected to repetitive injections, either with PBS, control MAb or therapeutic agents. Test agents were administered subcutaneously. Details about the time of tumor inoculation and dose of the injected agents are shown in Table 3-2. Intervals between each immunization and serum collection are shown in Table 3-3 and Table 3-4. All test antigens were injected along with Quil A.

Serum collection

Serum was routinely collected between each immunization as indicated in Table 3-3, 3-4. The tail of each mouse was nicked and the blood was collected in serum separator microcontainers. The blood was centrifuged at 300x g for five minutes. Resulting serum was frozen at –20°C for later testing.

Table 3-2 Characteristics of the Induction Anti-PSA Immune Response *in vivo*

Mice	Tumor cell Inoculum	Tumor Administration Route	Test Agents	Dose of Testing Agents (μg/mouse)
Balb/c	Line 1-PSA 0.05×10^6	iv.	Anti-PSA MAb Anti-PSA MAb + PSA 33D1 MAb 33D1 conjugate 33D1 conjugate + PSA DEC-205 MAb DEC-205 conjugate DEC-205 conjugate+PSA PSA PBS	MAb 50 μg Conjugates 100 μg PSA 10 μg Quil A 10 μg
DBA	P815-PSA 0.4×10^6	sc.		

Detection of the anti-PSA immune response by ELISA

To detect the anti-PSA immune response, ELISA was applied to measure anti-PSA Abs in the collected mouse sera. Microtitre strips were coated with PSA at a concentration of 0.5μg/mL, 100μl per well. The strips were incubated overnight at 4°C. The strips were then blocked with 150μl of blocking buffer for 1 hour, followed by rinsing with PBST. Sera, 1:50 diluted, were then added and the strips were incubated for 1 hour at room temperature with gentle shaking. The following procedure was the same as that described in Section 3.2.3.2.

Table 3-3 Schedule of *in vivo* Anti-PSA Immune Response Induction in Balb/c Mice

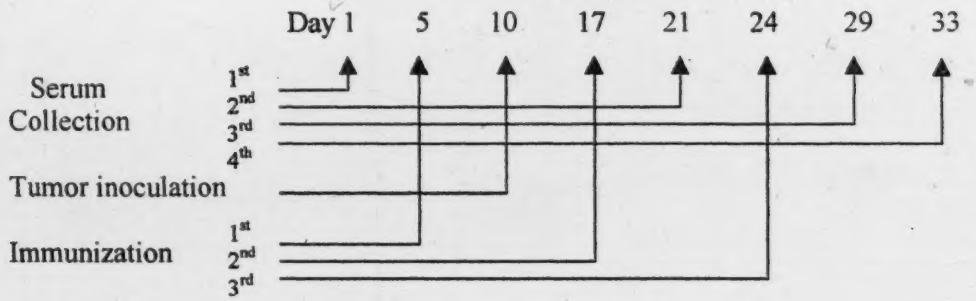
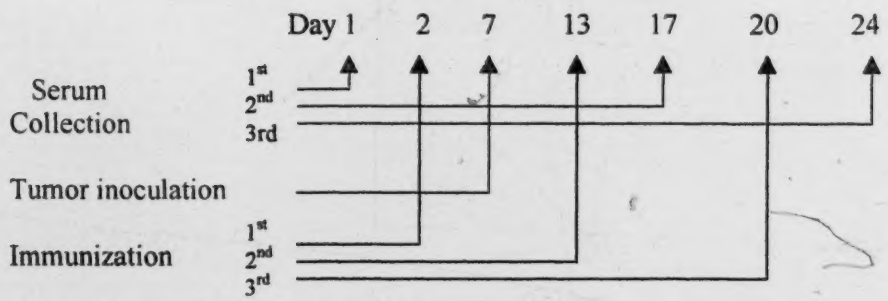


Table 3-4 Schedule of *in vivo* Anti-PSA Immune Response Induction in DBA Mice



3.2.3.9. Assay of ^{125}I labeled PSA uptake assay by murine dendritic cells

Iodination of PSA

PSA solution, 100μg in 100μL PBS, was pipetted into a vial. Approximately 600μCi of ^{125}I was added. The vial was gently shaken, and then 10μL of 2.5% chloramine-T was added. The vial was shaken for an additional 30 seconds. A 10μL aliquot of 5% sodium metabisulfite was added to stop the reaction. After 15 seconds, 20μL of 1M cold sodium iodide solution was added, and mixed thoroughly for 5 seconds. The contents of the vial were then loaded on a 10DG desalting column which was prewashed with 60mL of 0.5% BSA. The vial was rinsed with 0.5mL PBS and the rinse was loaded on the column. The column was then washed with PBS and the fractions were collected in Eppendorf vials, 0.5mL per fraction. Fractions with significant radioactivity (detectable by Geiger counter set on 100x of reading) were pooled. The radioactivity of ^{125}I-labeled PSA was measured on a dose calibrator.

Modification of iodinated PSA

^{125}I-PSA was mannosylated and mannan-conjugated. The methods were the same as those described in Section 3.2.2.2.

Assay of ^{125}I-labeled PSA uptake assay by murine dendritic cells

The dendritic cell line was cultured in Complete Growth Medium, which was α-MEM with ribonucleosides and deoxyribonucleosides, containing 4mM L-glutamine, 1mM sodium pyruvate, and 5ng/mL murine GM-CSF. The medium was supplemented with

20% FBS. Only the murine dendritic cells in suspension were taken for this experiment. The cells were washed twice with PBS and centrifuged at 360x g. The cell suspension was adjusted to 4 x 10^5cells/mL. A 0.5mL cell suspension was added to each vial. Mannosylated, mannan-conjugated ^{125}I-PSA, as well as ^{125}I-PSA (as a control group) were added and incubated for 15, 45, 80, 120 minutes, at 37°C. Mannose, 100μg/mL in PBS, was used as inhibitor with mannosylated ^{125}I-PSA as a control group. Approximately 80,000cpm ^{125}I-modified PSA was added to each vial. Each group had four replicates. After incubation at the time intervals indicated above (15, 45, 80, 120 min), the vials were centrifuged at 360x g for 5 minutes. The cells were then washed three times with PBS. The radioactivity retained in the cell pellets was measured with a 1480 WIZARD 3" Automatic Gamma Counter (Fisher Scientific Ltd. Nepean, ON, Canada).

CHAPTER 4

Summary, Conclusions and Future Work

An anti-PSA MAb hybridoma was generated previously in our lab with the aim of developing an effective immunotherapeutic agent for prostate cancer. Preliminary studies demonstrated that anti-PSA MAb produced by this hybridoma had strong binding affinity to human PSA. The MAb also slowed PSA-expressing tumor progress in animal experiments. In an attempt to increase the capacity of the MAb for the induction of anti-PSA immune response against prostate cancer, three basic experiments were conducted: 1. Modification of PSA and anti-PSA MAbs. 2. Characterization of modified PSA and anti-PSA MAbs. 3. Induction of specific immune response by modified PSA and anti-PSA MAbs *in vitro* and *in vivo*.

In order to obtain sufficient quantities of MAbs for the study, large-scale production of the anti-PSA MAb and anti-DC MAbs (hybridomas purchased from ATCC) was performed. From the well-studied anti-PSA MAb hybridoma, which is of murine origin, large quantities of anti-PSA MAb were obtained directly from Balb/c mouse ascites. For anti-DC MAb hybridomas (N418, DEC-205, 33D1), which are of non-murine origin, (DEC-205 and 33D1 from rat, N418 from hamster), large scale hybridoma cell culture in different cell culture media (standard medium, UD, SFM) and ascites production in SCID beige mice were performed. There was no significant difference in anti-DC MAb production from the different cell culture media, and protein impurities existing in final products of purified anti-DC MAbs were low enough to be considered as minor

contaminants. Ascites production in SCID beige mice varied with each clone. N418 and 33D1 hybridomas didn't generate sufficient volume of ascites (about 1.0 ~ 5.0mL per mouse) or concentration of MAb (25.6μg/mL for N418, 17.6μg/mL for 33D1) to be useful. DEC-205 hybridoma produced high MAb concentration in ascites (3130μg/mL) although with low volume of ascites (1.0mL). For the future study of these anti-DC MAbs, economic standard medium can be applied in cell culture for large-scale MAb production. Generation of ascites in SCID beige mice for DEC-205 MAb production may also be a viable alternative.

High purity MAbs were successfully obtained by protein G affinity chromatography. Protein G chromatography is a very reliable method with high efficiency for the purification of these MAbs. The column can be reused over a long period, thus lowering production costs. Final products contained very low levels of contaminants and the range of purification yield is from 54-100%.

Bispecific agents with binding activity to PSA as well as to DC were chemically generated by using the cross linker, glutaraldehyde to conjugate anti-PSA MAb and anti-DC MAb. This chemical conjugation method did not require a complicated procedure. The major disadvantage of this method is that the cross linker is nonspecific, thus, the final product is a mixture of conjugates of any two MAbs. In addition, the antigen binding sites of the MAbs can be involved in cross-linking thereby reducing the binding affinity of the conjugated product. PSA binding activity of the bispecific agents was tested by ELISA. Results showed that the bispecific agents retained some PSA binding

activity at concentrations greater than 300ng/mL, and the more glutaraldehyde used, the lower PSA binding activity obtained.

DC binding activity of the bispecific agents was determined by FACS analysis. DC were freshly isolated by collagenase digestion method. DC were further enriched by the plastic adherence method in the presence of GM-CSF. There were no significant differences in binding activity between bispecific agents and single anti-PSA or anti-DC MAbs. Induction of the anti-PSA immune response was tested *in vivo* in Balb/c and DBA mice challenged with Line1-PSA and P815-PSA tumor cell lines, respectively. ELISA was applied for detection of anti-PSA Abs in mouse sera. In the bispecific agent group, anti-PSA Ab could be detected compared with negative control groups (anti-DC MAb alone group and PBS group).

To maintain binding activity of the conjugates to PSA and DC, the conjugation methods will need to be improved. Linkers with binding specificity will be a better choice to protect antigen binding sites of MAbs. Purification of the final product is essential. Also, biological technology may be a choice to produce bispecific agents, such as somatic fusion of two hybridomas secreting the desired antibodies [Suresh MR, 1986], which maintain both PSA and DC binding activity. Such conjugates may demonstrate that bispecific agents have the ability to increase capacity to induce a specific immune response against PSA.

We attempted to achieve higher anti-PSA MAb presentation on DC in order to induce a more efficient anti-PSA immune response. Mannosylation of anti-PSA MAb was therefore studied. The mannosylation method was easy to manipulate. By monitoring fractions from Con A chromatography of mannosylation products by ELISA/UV, the optimal reaction condition was determined to be using 500-fold excess of α-D-M. Anti-PSA MAb could be fully mannosylated under this condition. The binding activity of mannosylated anti-PSA MAb towards PSA and DC was assessed by indirect ELISA and fluorescence microscopy. PSA binding was highly inhibited by mannosylation. Results showed no significant differences between mannosylated and nonmannosylated anti-PSA MAb on DC binding. The Fc receptor on DC may play a role by interference the uptake of mannosylated anti-PSA MAb. To confirm the ELISA results, the sandwich assay for PSA binding activity should be tested to obtain better evaluation of the binding activity of mannosylated anti-PSA MAb. Also, induction of anti-PSA immune response by mannosylated anti-PSA MAb and mannosylated PSA were tested *in vitro* (by Dr. Berlyn, University of Maryland, USA). There was no difference with mannosylated anti-PSA MAb compared with nonmannosylated anti-PSA MAb, but mannosylated PSA could induce a much stronger humoral immune response than nonmannosylated PSA.

Apostolopoulos V and his colleagues have published a series of papers on the specific CTL immune response induced by oxidized mannan conjugated proteins (Muc1) compared with reduced mannan conjugated protein *in vivo* [Apostolopoulos V, 1996]. Mechanistic studies *in vitro* revealed that oxidized mannan conjugated protein targets the MHC class I antigen-presentation pathway, and the effect was 'mannose receptor-

mediated [Apostolopoulos V, 2000]. In this thesis, oxidized mannan conjugation on anti-PSA MAb and PSA were successfully conducted, with the ultimate goal of inducing a PSA specific CTL immune response against PSA. Mannan conjugated anti-PSA MAb retained its PSA binding activity, which was evaluated by sandwich ELISA. Although no further studies were performed on mannan conjugated anti-PSA MAb, the residual PSA binding activity suggests that mannan conjugated anti-PSA MAb has potential for further studies on the induction of a specific anti PSA immune response.

Human CTLs specific for PSA have been generated *in vitro*, and the induction of an anti-PSA immune response in an animal model can protect the animals from subsequent tumor challenge [Correale P, 1997, 1998; Xue BH, 1997]. PSA has become a suitable target antigen for immunotherapy for prostate cancer. In the experiments described in this thesis, mannosylated PSA and mannan conjugated PSA were successfully prepared and characterized. Mannosylated PSA bound to DC at significantly higher levels than nonmannosylated PSA, as assessed by fluorescence microscopy. Also, mannosylated PSA could induce much stronger humoral immune responses *in vitro* (conducted by Dr. Berlyn). The *in vitro* DC uptake of mannosylated and mannan-conjugated PSA labeled with ^{125}I was compared to nonmodified PSA. Mannosylated PSA had higher uptake by DC. Optimization of the DC uptake assay needs to be done in areas, such as the choice of DC source, and the ideal incubation parameters. The use of other sources of DC such as bone marrow or peripheral blood might be an improvement. Bone marrow contains more DC precursors and would be a better source of DC than a long-term cultured DC cell line. Applying a fluorescent tag as an uptake indicator instead of the radioisotope ^{125}I, may be

an alternative. This way, the concentration of tested proteins can be increased to ensure that DC uptake is mainly mediated through mannose receptors. Purification of mannan conjugated ^{125}I-labeled PSA in order to get rid of free mannan is necessary to avoid competitive binding on mannose receptors.

Oxidized mannan conjugated PSA, mannosylated PSA and mannan conjugated anti-PSA MAb are the choices as future research objectives to fulfil the ultimate goal of this project to develop an immunotherapeutic agent for the treatment of prostate cancer.

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